Calcium Ion Promotes Yeast Dmc1 Activity via Formation of Long and Fine Helical Filaments with Single-stranded DNA*

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Dmc1 is specifically required for homologous recombination during meiosis. Here we report that the calcium ion enabled Dmc1 from budding yeast to form regular helical filaments on single-stranded DNA (ssDNA) and activate its strand assimilation activity. Relative to magnesium, calcium increased the affinity of Dmc1 for ATP and reduced its DNA-dependent ATPase activity. These effects, together with previous studies of other RecA-like recombinases, support the view that ATP binding to Dmc1 protomers is required for functional filament structure. The helical pitch of the Saccharomyces cerevisiae Dmc1-ssDNA helical filament was estimated to be 13.4 ± 2.5 nm. Analysis of apparently “complete” Dmc1-ssDNA filaments indicated a stoichiometry of 24 ± 2 nucleotides per turn of the Dmc1 helix. This finding suggests that the number or protomers per helical turn and/or the number of nucleotides bound per Dmc1 protomer differs from that reported previously for Rad51 and RecA filaments. Our data support the view that the active form of Dmc1 protein is a helical filament rather than a ring. We speculate that Ca2+ plays a significant role in regulating meiotic recombination.

Genetic and cytological data implicate Dmc1 and Rad51 in meiotic chromosome pairing, synapsis, and homologous recombination (1–3). Dmc1 is meiosis-specific, whereas Rad51 functions in both mitotic and meiotic recombination. Both proteins share the ability of RecA to promote DNA homology search and strand exchange in vitro (4–9). This activity is thought to account for the role of the two proteins in recombination reactions. However, the recombinogenic resolution of double strand breaks in vivo. Like the RecA protein, Rad51 proteins form helical nucleoprotein filaments and mediate homologous DNA pairing and strand exchange in an ATP-dependent manner in vitro (3, 4, 10). The HsDmc1 protein has been found to associate with ssDNA in the form of an octameric ring (6, 7, 11, 12). This discovery prompted the suggestion that the ring form of Dmc1 is functional during recombination. However, the recombinase activity of Dmc1 in these studies was quite low. In a more recent form of Dmc1 is functional during recombination. However, the recombinase activity of Dmc1 in these studies was quite low. In a more recent study, Sehorn et al. (9) reported short (~98 nm in length) but defined HsDmc1 helical nucleoprotein filaments under conditions that were favorable for recombination. This finding led to the proposal that the functional form of Dmc1 is a helical filament, consistent with other members of the RecA family of recombinases.

Like the human protein, Dmc1 from budding yeast (ScDmc1) exhibits low recombinase activity in vitro when expressed in and purified from Escherichia coli cells (8, 13, 14). Using atomic force microscope (AFM) with carbon nanotube tips (CNTs), we recently showed that the ScDmc1 protein alone also forms octameric rings. In addition, in the presence of 1 mM Mg2+ and 1 mM adenylyl-imidodiphosphate (AMP-PNP), 90% of ScDmc1 protein bound ssDNA as protein rings and only 10% was able to form a filamentous nucleoprotein structure (14). The results presented below indicate that substituting Ca2+ for Mg2+ enhances the biochemical activity of the protein in recombination reactions. This increase in activity is associated with an increased tendency of the protein to form regular helical filaments on DNA. These findings support the view that ScDmc1 becomes catalytically active only when it forms a helical nucleoprotein filament with ssDNA (9, 14) and raise the possibility that Dmc1 activity is modulated by Ca2+ in vivo.

MATERIALS AND METHODS

Proteins and DNA—The ScDmc1 protein was purified as described previously (8). FX174 ssDNA was purchased from New England Biolabs. In this study, the DNA concentrations are expressed as moles of nucleotides.

Strand Assimilation (“D-loop”) Assay—The ability of Dmc1 to promote homologous strand assimilation was determined by homolog-dependent association of oligonucleotide and supercoiled plasmid using a method similar to that described previously (8). In this assay, Dmc1 is first bound to ssDNA oligonucleotide, and then a homolog-containing super-coiled target plasmid is added. Standard Dmc1-oligo binding reactions were 15 μl and contained Dmc1 (0.54 μM), 32P-labeled oligonucleotide 306.7 (1.0 μM) (8), 20 mM HEPES (pH 7.5), 1 mM dithiothreitol, 2 mM ATP (or AMP-PNP), and 1 mM divalent cation. Reaction mixtures were incubated for 5 min at 37 °C to allow the formation of Dmc1-ssDNA complexes. After preincubation, 10 μl of 4.6 μM supercoiled target plasmid DNA (pRS307) in 23.5 mM divalent cation was added each binding reaction (final divalent cation concentration of 10 mM). Samples were further incubated to allow strand assimilation to occur before being stopped by the addition of SDS and proteinase K to final concentrations of 0.08% and 0.16 mg/ml, respectively. Samples were further incubated for 30 min at 37 °C. DNA was then separated on 1.3% agarose gels for 1.5 h at 105 V. Gels were squashed onto nylon membranes using a stack of paper towels and a 0.5-kg weight. Gels were analyzed by phosphorimaging (Molecular Dynamics) with the efficiency of D-loop formation given as the ratio of counts co-migrating with pRS307 over the total counts in each lane. The method gives a

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3 The abbreviations used are: ssDNA, single-stranded DNA; AFM, atomic force microscopy; AMP-PNP, adenylyl-imidodiphosphate; CNT, carbon nanotube; FWHP, full width at half-peak; Hs, Homo sapiens (protein designation prefix); Sc, Saccharomyces cerevisiae (protein designation prefix).
**RESULTS**

**Calcium Enhances ScDmc1-mediated Homologous Strand Assimilation**—A cursory analysis indicated that budding yeast Dmc1 can form D-loops more efficiently in the presence of Ca\(^{2+}\) than in the presence of Mg\(^{2+}\) (15). We further characterized this effect by carrying out time course analysis under two conditions (Fig. 1). First, we used conditions previously optimized for product yield in the presence of Mg\(^{2+}\). In these reactions, the concentration of divalent cation was increased from 1 to 10 mM when target duplex DNA added. Under this condition, the initial rate and final yield of product formation were ~5-fold greater in Ca\(^{2+}\) than in Mg\(^{2+}\) (Fig. 1). The second condition retained divalent concentration at 1 mM following target addition. This condition was chosen because it is the same as that used to examine the structure of Dmc1-ssDNA complexes in our previous structural study (14) as well as in the structural experiments described below. When divalent cation was retained at 1 mM, the difference in the efficiency of assimilation was even more dramatic than in the previous condition; no assimilation was detected with Mg\(^{2+}\), whereas Ca\(^{2+}\) supported a level of D-loop formation only ~2-fold less efficient than that seen with the higher divalent concentration. Given that intracellular free Ca\(^{2+}\) and Mg\(^{2+}\) levels in *Saccharomyces cerevisiae* had been estimated to range from 0.3 mM to 1 mM and from 0.1 mM to 1 mM, respectively (16, 17), these findings suggest that the lower concentrations are more physiological and, therefore, that Ca\(^{2+}\) is more likely than Mg\(^{2+}\) to be the relevant cofactor for Dmc1 activity in vivo.

The ssDNA-dependent ATPase Activity of ScDmc1 Is Lower in Ca\(^{2+}\) than in Mg\(^{2+}\)—To shed light on the molecular mechanism underlying Ca\(^{2+}\) activation, we examined the ssDNA-stimulated ATPase activity of ScDmc1 in the presence of Ca\(^{2+}\) or Mg\(^{2+}\) (Fig. 2). ATPase activities were determined by the release of \(\gamma\)-\(^{32}\)P from \(\gamma\)-\(^{32}\)P-ATP. The rate of hydrolysis was 3-fold lower in the presence of 1 mM Ca\(^{2+}\) \((k_{\text{cat}} \sim 0.32 \pm 0.01 \text{ min}^{-1})\) than in the presence of 1 mM Mg\(^{2+}\) \((k_{\text{cat}} \sim 1.05 \pm 0.06 \text{ min}^{-1})\). Importantly, we found that the ScDmc1 protein exhibited higher binding affinity for ATP in the presence of Ca\(^{2+}\) \((K_{\text{m}} = 0.34 \pm 0.024 \text{ mM})\) than in the presence of Mg\(^{2+}\) \((K_{\text{m}} = 1.03 \pm 0.10 \text{ mM})\). A low level of Ca\(^{2+}\) \((0.1 \text{ mM})\) in the presence of 0.9 mM Mg\(^{2+}\) resulted in a significant increase in ATP binding \((K_{\text{m}} = 0.62 \pm 0.10 \text{ mM})\) and a reduction in ATP binding and targeting phases of the reaction.

**Fig. 1.** Comparison of the efficiency of DNA strand assimilation activity of ScDmc1 in the presence of Mg\(^{2+}\) or Ca\(^{2+}\). Time course analysis of D-loop formation was carried out as described under “Materials and Methods.” A, phosphorimage of an agarose gel of time points taken from D-loop reactions carried out in the presence of Mg\(^{2+}\) and Ca\(^{2+}\) as indicated. B, quantitation of D-loop time course experiments. Squares, reactions in the presence of Ca\(^{2+}\); diamonds, reactions in the presence of Mg\(^{2+}\); solid lines, divalent cation concentration increased from 1 to 10 mM when target duplex DNA added; dashed lines, divalent cation concentration at 1 mM during both ssDNA binding and targeting phases of the reaction.
hydrolysis ($k_{cat} = 0.75 \pm 0.06 \text{ min}^{-1}$) as compared with 1 mM Mg$^{2+}$ (Fig. 2). These results indicate that Ca$^{2+}$ fosters the formation and stability of Dmc1-ATP-ssDNA complexes. The results are in agreement with previous observations indicating that the active form of recombinase filaments is the ATP-bound form and that ATP hydrolysis is not required for homology searching or strand assimilation (8, 18–20).

Substituting ATP with AMP-PNP Does Not Enhance Strand Assimilation Efficiency in the Presence of Ca$^{2+}$—Our previous work showed that, under conditions in which D-loop formation can be detected with Mg$^{2+}$, the non-hydrolyzable analogue AMP-PNP yielded more products than ATP. For this reason we were interested in determining whether AMP-PNP and Ca$^{2+}$ can stimulate D-loop activity independently. Under the increasing Ca$^{2+}$ condition we found the same level of D-loop formation with Ca$^{2+}$-ATP and Ca$^{2+}$-AMP-PNP (Fig. 3). The absence of any additive effect on stimulation is consistent with the possibility that AMP-PNP stimulates D-loop activity by a mechanism shared with Ca$^{2+}$. The most likely possibility is that this mechanism is the suppression of ATP hydrolysis. Although AMP-PNP can stimulate Dmc1 in the absence of Ca$^{2+}$, this stimulation is not as efficient as that conferred by Ca$^{2+}$. When divalent cation is increased to 10 mM during the targeting phase of the reaction, Mg$^{2+}$ plus AMP-PNP yielded only one-third of the level of D-loops as that obtained with Ca$^{2+}$ plus AMP-PNP or Ca$^{2+}$-ATP. These results imply that Ca$^{2+}$ can influence the efficiency of D-loop formation in a manner independent of its effect on ATP hydrolysis. This influence could be limited to the effect on nucleotide binding affinity described above. Alternatively, Ca$^{2+}$ could also have a nucleotide-independent influence on Dmc1 assimilation activity. When divalency was retained at 1 mM during D-loop reactions, the difference between the two nucleotides was even more dramatic; AMP-PNP supported little or no D-loop formation even in the presence of Ca$^{2+}$.

Calcium Ion Promotes ScDmc1 Proteins to Form Extensive and Regular Helical Filaments with ssDNA—AFM-CNTs was used to examine the effect of Ca$^{2+}$ on the structure of ScDmc1 filaments (Fig. 4A). We reported recently that the majority (~90%) of ScDmc1 bound ssDNA as rings in the presence of 1 mM Mg$^{2+}$ plus AMP-PNP using the AFM-CNT imaging method (14). Under these conditions, D-loop formation is extremely inefficient. In contrast, conditions that support a relatively high level of assimilation, 1 mM Ca$^{2+}$ plus ATP, yielded nucleoprotein filaments with regular diagonal striations indicative of right-handed helical structure (Fig. 4B).

Cross-section analysis was carried out by AFM-CNT method to determine both helical pitch and width (e.g. full width at the half-peak, FWHP) of the ScDmc1-(Ca$^{2+}$-ATP)-ssDNA helical filaments (Fig. 5A). The FWHP of ScDmc1 helical filament (13.5 ± 0.8 nm) is slightly wider than that (12.3 ± 0.4 nm) of RecA-ssDNA filaments. As described previously (14), the FWHP values of ssDNA and ScDmc1 ring-ssDNA were 6 and 18 nm, respectively. Fine ScDmc1 helical nucleoprotein filaments were also observed in the presence of 10 µM Ca$^{2+}$, 1 mM Mg$^{2+}$, and 1 mM ATP (data not shown), indicating that low concentrations of Ca$^{2+}$ can support formation of helices with the same helical pitches even when Mg$^{2+}$ is in 100-fold excess. We conclude that Ca$^{2+}$ promotes formation of helical filaments of ScDmc1 on ssDNA. This result supports the conclusion that Ca$^{2+}$ stimulates Dmc1-dependent D-loop activity by promoting the formation of helical filaments.

In cases where helical structure can be detected across a saturated nucleoprotein filament of known length, it is possible to calculate the number of nucleotides per helical turn of the filament (21). Three molecules were imaged in which the diagonal striations characteristic of helical structure could be seen across the entire length of the 872-nucleotide ssDNA molecule (Fig. 4, C–F). The three complete molecules contained 35, 38, and 40 striations. The contour length of these molecules was 465, 467, and 472 nm, respectively. Striation counts can only be taken to reflect helical stoichiometry if recombinase protomers do not extend past the end of the DNA. Given the similar contour lengths and narrow range of striation counts in the favorable molecules, we feel it is reasonable to assume that the extension of Dmc1 protomers beyond...
DNA ends was limited and/or did not occur for the three molecules examined. Under this assumption, the number of striations per DNA filament suggests that there are 24 nucleotides per turn of the helical filament. This number is surprising in that RecA has been shown to bind 18 nucleotides per helical turn (21, 22). The possible implications of this finding are discussed below.

Data collected from the three molecules described above, as well as from an additional 12 molecules displaying substantial helical segments, were used to determine the average pitch of the helix. The helical pitch was found be 13.4 ± 2.5 nm (n = 171, Fig. 5B). This number suggests that the contour length of ssDNA in the helical Dmc1 filament is similar to that of a single strand in duplex DNA that is extended ~1.6 ± 0.3-fold relative to B-form DNA. Consistent with this finding, the total length of 872-nucleotide ScDmc1-ssDNA filament, the ATP-ssDNA helical filaments, is 468 ± 2 nm. The degree of DNA extension seen for Dmc1-ssDNA filaments is identical to that reported previously for RecA (21, 22) and was confirmed by us using the same AFM conditions identical to those for ScDmc1.4 Thus, Dmc1 and RecA appeared to extend ssDNA by about the same amount but underwind it to different degrees, namely

4 Y.-C. Chang, C.-S. Chang, and T.-F. Wang, unpublished observations.

FIGURE 4. Ca2+ promotes formation of ScDmc1-ssDNA helical filaments. A, AFM-CNT visualization of the ScDmc1-ssDNA nucleoprotein helical filaments (a, b, and c). The ScDmc1 protein was incubated with an 872-nucleotide DNA fragment as described under “Methods and Materials.” B, magnification of ScDmc1 helical filament segments (a, b, and c), which were marked with arrows in panel A. All of these helical filaments are right-handed. C, low resolution AFM images of three ScDmc1-ssDNA helical filaments with the entire length of 872 nucleotides. These three molecules were marked as d (472 nm), e (467 nm), and f (470 nm), respectively. D, high resolution AFM images of the ScDmc1-ssDNA helical filament (d) before (top) and after (middle) phasing analysis using the microscope’s analysis software. Cross-section analysis indicates that this ScDmc1-ssDNA filament contains 40 helical striations (bottom). E, cross-section scanning analysis of the third ScDmc1-ssDNA helical filament (e). The total helical pitch number of this molecule is 38. F, cross-section scanning analysis of the third ScDmc1-ssDNA helical filament (465 nm). The total helical pitch number of this molecule is 35.

FIGURE 5. Histogram analysis of the FWHP values of different AFM images and the helical pitches of ScDmc1-ssDNA helical filament. A, cross-section analysis were carried out to determined FWHP values and helical pitches. FWHP was used to avoid overestimation of width. The y-axis shows the FWHP. The x-axis shows the relative distribution frequency of different FWHP values for ssDNA (n = 325), RecA-ssDNA helical filaments (n = 510), and ScDmc1-ssDNA filaments in the presence of AMP-PNP and Mg2+ (n = 674) or ATP and Ca2+ (n = 623). Data for RecA and Dmc1 in the presence of Mg2+ was published previously (14) and is presented here with permission from the journal Biochemistry and the American Chemical Society. B, histogram analysis of helical pitches of ScDmc1-ssDNA filaments in the presence of Ca2+.
**Ca^{2+} Promotes Yeast Dmc1 Function**

Dmc1 to 24 nucleotides per turn and RecA to 18 nucleotides per turn (21, 22).

**DISCUSSION**

The results described above contribute to the unifying view that all recombinases of the RecA family function as right-handed helices on ssDNA and argues strongly against the proposal that Dmc1 promotes D-loops via a toroid-mediated interaction. Ca^{2+} promoted ScDmc1 helical filament formation and D-loop activity. From this result, we speculate that the presence or absence of Ca^{2+} in reagents (e.g. ATP-regenerating systems, buffers, and salts) used in previous reports (6, 7, 9) may explain (at least partially) why purified HsDmc1 from different laboratories behaved differently. A very recent report on the human Dmc1 protein supports this notion (see below) (23). Intriguingly, Ca^{2+} may explain (at least partially) why purified HsDmc1 from different regenerating systems, buffers, and salts) used in previous reports (6, 7, 9) indicated that Ca^{2+} is a general modulator for DNA exchange promoted by both yeast and mammalian homologous recombination proteins. However, there is obvious similarity between Rad51 and Dmc1 in that in both cases the ATPase activity is lower in Ca^{2+} than in Mg^{2+}.

For filaments in which it was possible to count helical turns across the entire length of a coated DNA, we calculated a stoichiometry of ~24 nucleotides per helical turn. This finding differs from values obtained for RecA, which binds 18 nucleotides per helical turn. RecA filaments have six protomers per turn and, hence, three nucleotides per protomer (21, 22). Although we cannot exclude the possibility that the higher ratio reflects helical assembly of Dmc1 beyond DNA ends, the value suggests two interesting alternative possibilities. First, it is possible that there are eight Dmc1 protomers per helical turn. This possibility seems consistent with the fact that Dmc1 forms octameric rings, whereas the rings formed by RecA and Rad51 are hexamers (6, 7, 11, 12, 14, 18). Our finding that the width of Dmc1 filaments was slightly greater than that of RecA filaments viewed under the same conditions is also consistent with the idea that there are more monomers per turn, especially given the pitch of the Dmc1 filament (Fig. 3). Alternatively, it is possible that ScDmc1 filaments contain six protomers per turn, with each protomer binding four rather than three nucleotides. Studies of Dmc1 binding do not reliably distinguish between the possibilities of three versus four nucleotides per monomer (8). Although we favor the first possibility, further work is required to fully define the structure of Dmc1 helical filaments on DNA.

Finally, a previous study showed that Ca^{2+} ion influx occurs during budding yeast meiosis (26). Given this finding, the results presented here raise the intriguing possibility that the progress of meiotic recombination reactions is controlled by changes in intracellular calcium levels.

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**Addendum**—Following initial submission of this manuscript we became aware of independent work on human Dmc1 protein by A. V. Mazin and colleagues (23). These investigators showed that, like the protein from budding yeast, the ability of human Dmc1 to form helical filaments and D-loops is stimulated by calcium. It appears therefore that the influence of calcium on Dmc1 structure and function is a conserved feature of the protein.

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