

Human Rad50/Mre11 Is a Flexible Complex that Can Tether DNA Ends

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Summary

The human Rad50 protein, classified as a structural maintenance of chromosomes (SMC) family member, is complexed with Mre11 (R/M) and has important functions in at least two distinct double-strand break repair pathways. To find out what the common function of R/M in these pathways might be, we investigated its architecture. Scanning force microscopy showed that the complex architecture is distinct from the described SMC family members. R/M consisted of two highly flexible intramolecular coiled coils emanating from a central globular DNA binding domain. DNA end-bound R/M oligomers could tether linear DNA molecules. These observations suggest that a unified role for R/M in multiple aspects of DNA repair and chromosome metabolism is to provide a flexible, possibly dynamic, link between DNA ends.

Introduction

DNA double-strand breaks (DSBs) are necessary intermediates in a number of normal aspects of DNA metabolism, such as V(D)J recombination and meiosis (Modesti and Kanaar, 2001). In addition, DSBs can be caused by endogenous or exogenous DNA damaging agents. Unrepaired DSBs can be lethal, whereas misrepaired DSBs can cause chromosomal fragmentation, translocations, and deletions. Eukaryotic cells primarily repair DSBs by one of two distinct pathways, nonhomologous end-joining or homologous recombination (Baumann and West, 1998; Kanaar et al., 1998; Karran, 2000; Sung et al., 2000). These two pathways process and repair DSBs in different ways, but both require a protein complex containing Rad50, Mre11, and Nbs1 (or Xrs2 in yeast) (Ogawa et al., 1995; Dolganov et al., 1996; Trujillo et al., 1998; Usui et al., 1998; Paques and Haber, 1999; Paull and Gellert, 2000; Lewis and Resnick, 2000). The essential role of these gene products in mammals is underscored by the fact that disruption of *RAD50*, *MRE11*, or *NBS1* genes results either in cell inviability

or embryonic lethality (Xiao and Weaver, 1997; Luo et al., 1999; Zhu et al., 2001). Furthermore, mutations in the human *MRE11* and *NBS1* genes cause the cancer predisposition syndromes ataxia telangiectasia-like disorder and Nijmegen breakage syndrome, respectively (Carney et al., 1998; Stewart et al., 1999). A unified function for the Rad50, Mre11, and Nbs1 proteins in the distinct DSB repair pathways has not yet been identified.

One clue toward understanding the function(s) of the Rad50/Mre11 complex (hereafter referred to as R/M) comes from sequence analysis which places Rad50 in the structural maintenance of chromosomes (SMC) family of proteins (Sharples and Leach, 1995; Saitoh et al., 1995; Aravind et al., 1999; Strunnikov and Jessberger, 1999). The paradigm proteins in this class are involved in DNA condensation and structural chromosome maintenance, and they all have an interesting predicted architecture consisting of globular amino (N)- and carboxy (C) termini separated by an extended coiled-coil domain (Strunnikov and Jessberger, 1999; Fousteri and Lehmann, 2000; Holmes and Cozzarelli, 2000). This architecture has been beautifully confirmed by electron microscopy (EM) studies of the bacterial family members *E. coli* MukB, *Bacillus subtilis* SMC (Melby et al., 1998) and *E. coli* SbcCD (Connelly et al., 1998). For *E. coli* MukB and *B. subtilis* SMC, the coiled-coil domain has been shown to contain an antiparallel arrangement of two protomers with a flexible central hinge (Melby et al., 1998). It has been suggested that this structure has specific relevance for chromosome condensation by binding distant DNA sites with the terminal globular domains and effecting rearrangement of the intervening DNA through movement of the hinge (Melby et al., 1998; Holmes and Cozzarelli, 2000). Atomic level structure determination of the catalytic domain of two structural Rad50 homologs from archaea and bacteria confirmed the association of N- and C-terminal domains to form a functional ATPase active site (Hopfner et al., 2000; Lowe et al., 2001). Additional data from the crystal structure of the *P. furiosus* Mre11 and Rad50 structural homologs resulted in a model for the arrangement of Rad50 and Mre11 in a complex that is a variation of the general model for SMC proteins (Hopfner et al., 2001). Though the structural models of bacterial and archaeal SMC proteins and R/M homologs provide a starting point for understanding function, the structure of human R/M is not known, and differences from those already proposed could have important functional consequences.

We describe the architecture of human R/M and complexes of this protein with DNA using scanning force microscopy (SFM). SFM images showed human R/M to have a striking architecture that shares some features of, but is distinct from, the bacterial and archaeal SMC proteins so far described. Human R/M consisted of a large central globular domain from which two rod-like "arms" protruded. We observed a variety of conformations for the arms and demonstrated their remarkable flexibility by time-resolved SFM of single molecules in buffer. DNA was bound by the central globular domain of R/M. SFM images revealed preferential binding to

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linear DNA and accumulation of R/M oligomers at DNA ends. Different DNA molecules could be held together by interaction of the arms of the end-bound R/M oligomers. These data suggest that the architecture of R/M bound to DNA ends allows association of DNA through multiple interactions of the flexible arms. This ability to hold different DNA molecules together in a relatively nonspecific and flexible manner can explain the importance of R/M in the variety of DNA metabolic pathways.

Results

Human Rad50 Forms an Intramolecular Coiled Coil

To study the structure of human R/M, it was purified from Sf21 cells coinfecting with baculoviruses expressing histidine-tagged hRad50 and untagged hMre11. The identity of the proteins in the final fraction was confirmed by immunoblotting, and size fractionation confirmed that the purified hRad50 and hMre11 are present in a complex (data not shown). As shown for hMre11 alone, purified R/M bound to DNA and promoted annealing of complementary oligonucleotides (de Jager et al., 2001; data not shown). As expected, R/M also possessed an ATP-independent but manganese-dependent 3' to 5' exonuclease activity on blunt-ended double-stranded DNA substrates (Paull and Gellert, 1998; data not shown).

The amino acid sequence of hRad50 predicts that this protein would have a structure similar to bacterial SMC class molecules with N- and C-terminal globular domains connected by a 960 amino acid-long heptad repeat, likely to form a coiled-coil structure. We used SFM to determine the architecture of the purified human R/M complex. Human R/M indeed had a distinct architecture consisting of a large globular domain, from which two 40 to 50 nm arms protruded (Figure 1). The length of the arms was half of that expected for a 960 amino acid-long coiled-coil structure. To determine whether the individual arms consisted of a single hRad50 molecule folded back on itself or a complex of more than one coiled coil, we estimated the width of the arms. Dimensions of biomolecules measured by SFM are subject to distortions (Bustamante et al., 1993). Therefore, we compared the width of the arms to the width of DNA in the same image. Within the error of measurements, the R/M arms were about the same width as double-stranded DNA. Based on a width of 2 nm for B form DNA and 3 nm for a coiled coil, determined by X-ray crystallography (O'Shea et al., 1991), we believe the R/M arms are a single coiled coil.

The arms appeared in a variety of conformations varying from completely splayed (Figure 1B) to closed structures with the ends touching (Figures 1D and 1E). The individual arms appeared in straight, bent, and kinked conformations, which cannot simply be due to rotation around the globular domain. Open and closed arms appeared with a frequency of about 60% and 40%, respectively, which was not significantly influenced by the presence of Mg^{2+} , or Mn^{2+} and/or ATP.

R/M appeared in different oligomeric forms (Figure 1). The most abundant form (80% of all molecules) consisted of a single globular domain with two arms (Figures 1B–1E), which we refer to as a “monomer” complex. We never observed a globular domain with only one arm.

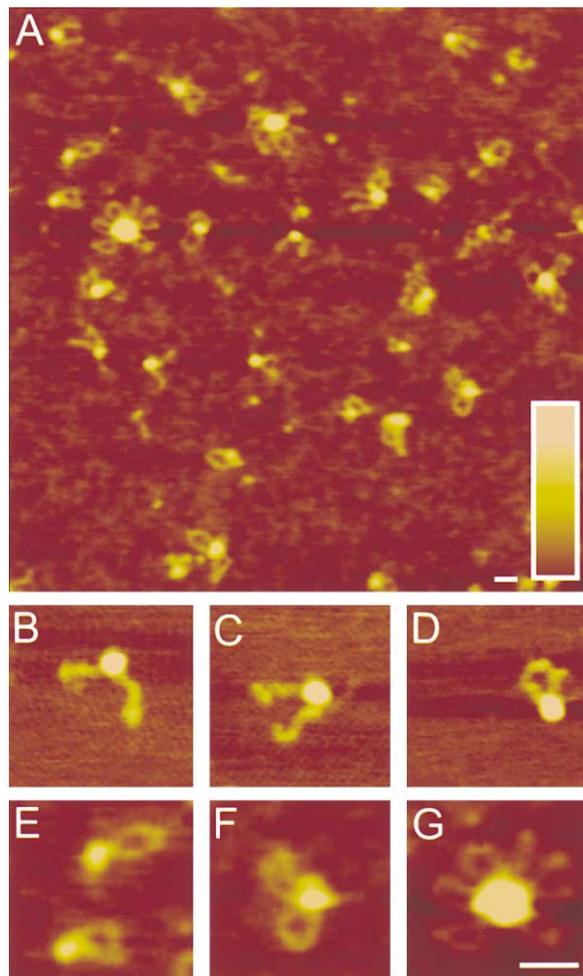


Figure 1. SFM Analysis of Human R/M

Purified R/M was deposited on mica and imaged by tapping mode SFM in air. (A) A typical field of complexes ($1 \mu\text{m} \times 1 \mu\text{m}$ scan). R/M exhibited a distinct architecture with a central globular domain from which arms of 40 to 50 nm protruded. The arms were observed in a variety of conformations (B–E) and multimeric forms (F and G). The scale bars are 50 nm. Color represents height from 0 to 1 nm (dark to light), as shown by the key insert in (A).

Though in some cases the arms were almost parallel or almost on top of each other, there were always clearly two arms. Based on the width and length of the arms and the composition of an archaeal R/M complex (Hopfner et al., 2001), we believe this form contains two hRad50 molecules and two hMre11 molecules. Other oligomeric forms were dimer complexes having four arms (14% of all molecules; Figure 1F) and some larger multimers, usually consisting of 4 to 6 monomers (6% of all molecules; Figure 1G). Multimers always contained an even number of arms, confirming that the two-armed version (Figures 1B–1E) is the unit structure. Larger protein aggregates were only very rarely observed in the absence of DNA.

The Coiled-Coil Arms of the Human R/M Complex Are Flexible

The different conformations of the protein complex, observed by SFM imaging in air (Figures 1B–1E), suggested

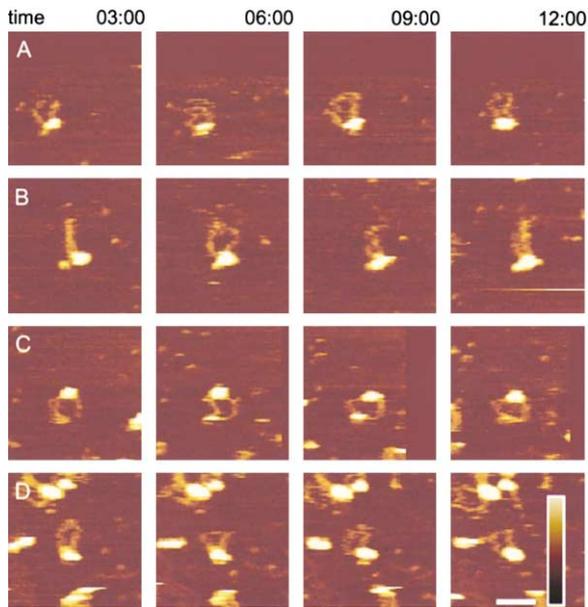


Figure 2. Time-Resolved SFM Imaging Reveals R/M to Be a Highly Flexible Structure

Purified R/M was deposited on mica and imaged by tapping mode in buffer. Each series of panels (A–D) shows a different single R/M complex observed over time. The panels are sequential images collected at 3 min intervals. The scale bar is 50 nm. Color represents height from 0 to 5 nm (dark to light), as shown by the key insert at the bottom right.

flexibility of the R/M arms. However, the different conformations observed could also be due to the presence of a mixture of static forms. In order to see if individual molecules were flexible, we used SFM imaging of partially immobilized R/M in buffer. This method allows time-resolved analysis of single molecules. Figure 2 shows four consecutive frames, taken at 3 min intervals, of four individual molecules. The molecules were only partially immobilized and moved during the time it took to collect an image; thus the images appear less clear. However, it was obvious that each individual R/M molecule adopted a variety of conformations over the time intervals measured. Both joining and unjoining of the tips of the arms (Figures 2A and 2D), as well as changes in curvature (Figures 2B and 2D), were observed for individual molecules, showing that the arms were indeed highly flexible. The flexibility of long polymer molecules can be expressed in persistence length (Rivetti et al., 1998). Though we do not know if the Rad50 arms can be accurately modeled as uniform polymers, as a first estimate of their flexibility, we determined their persistence length. The Rad50 arms have a calculated persistence length of 35 nm, making them more flexible than double-stranded DNA (persistence length 50 nm).

The Human R/M Complex Binds DNA Ends

The cellular roles of this protein and its relation to SMC proteins suggest that R/M could work by organizing broken DNA molecules for further processing. How R/M would do this will depend on which part of the protein binds to DNA and possible interactions between DNA-bound proteins. We used SFM to investigate the architectural features of R/M-DNA complexes. R/M bound

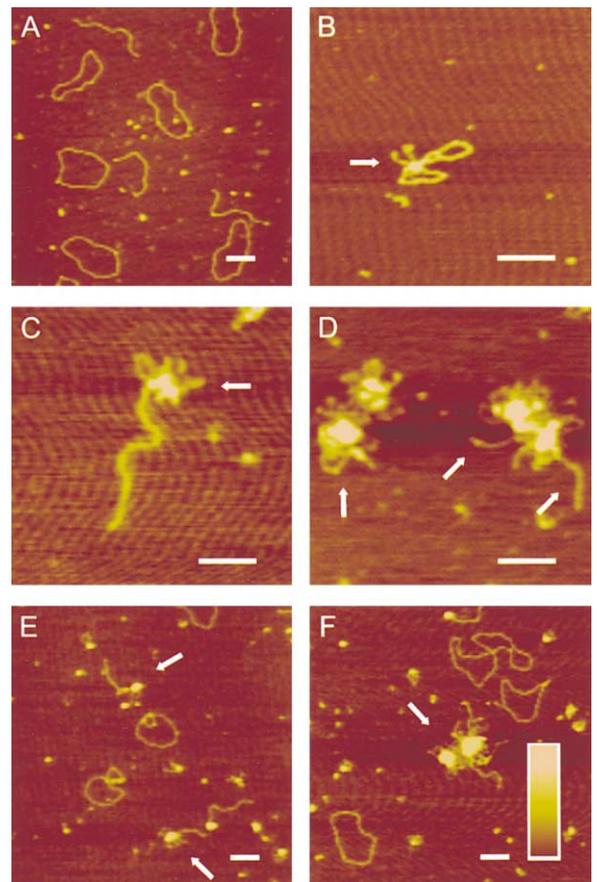


Figure 3. SFM Analysis of R/M-DNA Complexes

Reaction mixtures including the purified R/M and DNA were deposited on mica and imaged by tapping mode SFM in air. (A) The 1 kb linear and 1.8 kb circular DNA molecules that were used (either separately or together) in binding reactions. (B) R/M bound to the 1.8 kb circular DNA. (C and D) Reactions with 1 kb linear DNA at 15 nM of fragment, incubated with either 50 nM R/M (C) or 100 nM R/M (D). (E and F) Reaction mixtures including both the 1 kb linear DNA fragment and 1.8 kb relaxed circular DNA at 45 nM and 70 nM of fragments, respectively, incubated with 200 nM R/M complex. Arrows indicate protein molecules bound to DNA (B, C, E, and F) or DNA (D). The scale bars are 100 nm. Color represents height from 0 to 1 nm (dark to light), as shown by the key insert in (F).

to DNA via the globular domain, while the arms protruded from DNA (Figures 3B, 3C, and 3E). Significantly, DNA binding via the arms was not observed. On linear DNA, R/M bound almost exclusively to the ends (Figures 3C and 3D, and see below). Increased protein concentrations resulted in the accumulation of large R/M oligomers at DNA ends (Figure 3D compared to 3C). Although the protein oligomers could become quite large, and individual R/M molecules were difficult to resolve, in all cases the arms were extending away from the DNA. We did not observe objects with the height of the large globular domain at the periphery of the DNA-bound protein oligomers. At protein concentrations resulting in large R/M oligomers at DNA ends, we often observed tethering of DNA molecules (Figures 3D and 3F). Interestingly, tethering of DNA molecules appeared to occur via interactions of R/M arms and apparently required multiple contacts (see below).

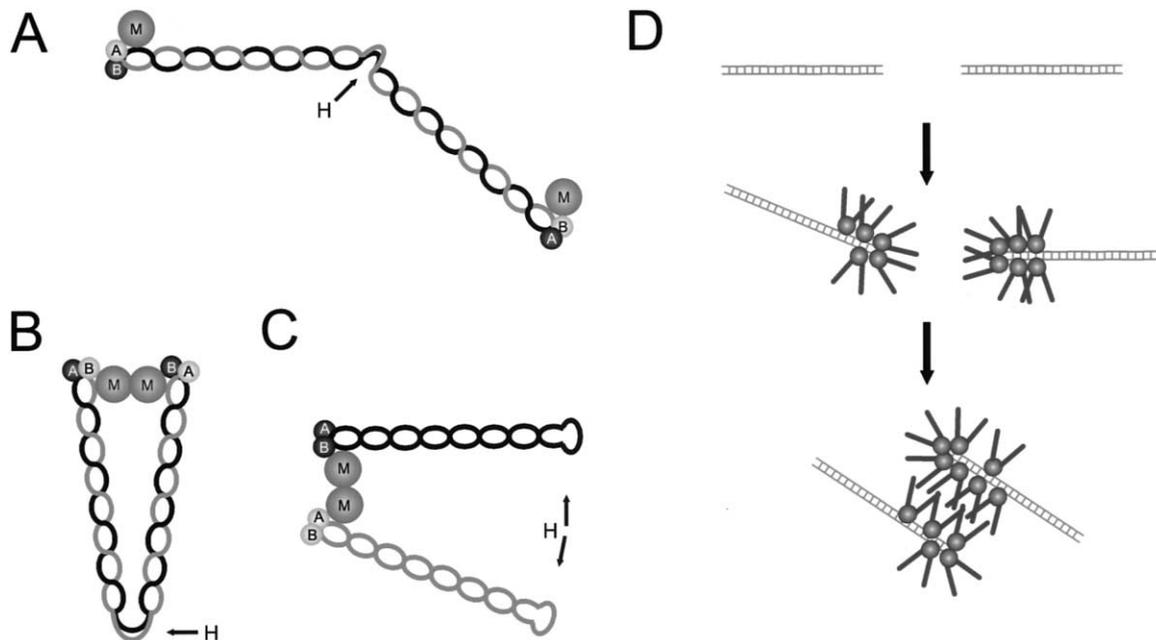


Figure 4. Possible Architectural Arrangements of Rad50 and Mre11 in the Complex and Implications for Its Roles in DNA Double-Strand Break Repair

(A) Hypothetical arrangement of R/M based on bacterial SMC proteins. Two antiparallel Rad50 molecules in an intermolecular coiled coil with distal ATPase domains, formed by combining an N- and C-terminal part of two different Rad50 molecules and Mre11 interacting with these domains.

(B) The arrangement proposed for the *P. furiosus* R/M structural homolog. Similar to (A) except that the two ATPase domains associate through an Mre11 dimer.

(C) Proposed arrangement for human R/M. Two folded Rad50 arms as intramolecular coiled coils associated with an Mre11 dimer. Rad50 molecules are represented as light and dark gray, with their two bipartite ATPase domains marked "A" and "B." Mre11 is represented as a gray sphere, marked "M." The putative flexible hinge region in the middle of the coiled coil is indicated by "H."

(D) Schematic representation of one of the hypothesized functions of R/M in DSB repair. R/M oligomers accumulate at broken DNA ends and keep the ends in close proximity by interaction of the end-bound R/M oligomers. The distinct architecture of the R/M complex could coordinate steps in end-processing and -joining by providing a flexible connection via multiple interaction sites. During nonhomologous end-joining, the R/M complex could provide a tether between the two ends of the same sister chromatid, while during homologous recombination, it could in addition provide a tether between a broken end and the intact sister chromatid.

To directly test if free DNA ends are a preferred substrate, we analyzed binding of R/M to a mixture of linear and circular DNA molecules. A preference for binding to DNA ends was not observed in gel retardation experiments (data not shown). For SFM, we used a molar ratio of 3:1 to 6:1 protein to DNA molecules. These protein concentrations are much lower than the minimal required to observe any DNA binding in a gel retardation assay. For SFM in air, the protein-DNA complexes present in the reaction mixture at the time of deposition are effectively frozen for imaging. Thus, it is possible to observe complexes by SFM that do not survive gel electrophoresis-based assays (Wyman et al., 1997). Using SFM, we observed a strong preference for binding of R/M to DNA ends (Figures 3E and 3F); 29% (105/357) of linear DNA was bound by protein versus 7% (43/624) for circular DNA. In addition, the complexes on circular DNA were very different from those on linear DNA. Almost all (92%) of the R/M complex bound to circular DNA was in its monomeric state. In contrast, about half (53%) of the linear DNA-R/M complexes included protein oligomers. In addition, multiple linear DNA molecules were often tethered together, accounting for 27% of the protein-bound DNA. We never observed circular

DNA molecules tethered together. Tethering of DNA molecules was not observed with one or a few R/M monomers. Association of DNA-bound R/M appeared to occur through interaction of the arms. Although individual arms at the junctions were not resolved, the protein in the obvious junction regions was not as high as the globular domains.

Discussion

Human R/M is an essential cellular component that functions in several aspects of DNA metabolism. R/M has direct roles in both major pathways of DSB repair, homologous recombination and nonhomologous end-joining (Paques and Haber, 1999; Chen et al., 2001 [this issue of *Molecular Cell*]). The amino acid sequence of Rad50 places it in the SMC class of proteins that in general affect chromosome condensation, DNA organization, and reorganization through the cell cycle. We have described the architecture of human R/M and elements of this architecture that are important for DNA binding. The ability to observe individual protein complexes in buffer allowed us to demonstrate the remarkable flexibility of the hRad50 coiled-coil domains. We

believe that our data indicate a general role for R/M in tethering DNA molecules together via multiple, presumably individually weak interactions of the flexible arms as a sort of molecular Velcro. Below, we discuss this model and compare this R/M architecture to that of other SMC proteins.

Our SFM images of human R/M reveal an architecture that is different from those so far proposed for SMC proteins or the bacterial and archaeal R/M structural homologs. This architecture has important implications for the interaction of R/M with DNA and between DNA molecules. The architecture of bacterial SMC proteins is shown in Figure 4A (Melby et al., 1998). This architecture is often presented as general for SMC proteins (Strunnikov and Jessberger, 1999; Hopfner et al., 2000). A variation of this, specific for archaeal and bacterial R/M structural homologs (Hopfner et al., 2000; 2001; Lowe et al., 2001), is shown in Figure 4B. Our proposed arrangement for human R/M, with intramolecular coiled coils, is shown in Figure 4C. We do not observe globular domains at the ends of the arms, ruling out the bacterial SMC-type architecture (Figure 4A). Qualitatively, the arrangement in Figure 4B could be possible for human R/M if the structures we observe consist of two such units. We do not believe this is the case for three reasons. First, we never see structures with one arm emanating from a globular domain. Second, the measured width of the arms is close to the measured width of DNA, likely representing one coiled coil. Third, biophysical characterization of the *P. furiosus* R/M homolog suggests that the unit form of the complex is R_2/M_2 (Hopfner et al., 2001). Interestingly, it has recently been demonstrated that altering the hinge region of *B. subtilis* SMC can convert this protein from an intermolecular dimer to intramolecular monomers, such as we propose here for human wild-type R/M (Hirano et al., 2001). EM images of yeast Rad50 and reconstituted Rad50/Mre11 have also been interpreted to support the model with intermolecular coiled coils shown in Figure 4B (Anderson et al., 2001). However, those data are also consistent with intramolecular coiled coils with associated hinge regions in the closed conformation that account for about 40% of human R/M molecules. In our proposed R/M structure, a functional ATPase is formed by interaction of N- and C-terminal domains from the same polypeptide folded in an intramolecular coiled coil. An Mre11 dimer is shown associated with Rad50 at the position predicted from the *P. furiosus* Rad50 and Mre11 structures (Hopfner et al., 2001). Though SFM does not provide information on the protein interfaces, because Mre11 itself forms dimers and based on the *P. furiosus* Mre11 and Rad50 interactions (Hopfner et al., 2001), we have indicated dimerization of the complex via Mre11.

The architecture of R/M has important implications for its interactions with DNA. In contrast to the proposed direct connection of DNA molecules by a single coiled coil of an SMC-type molecule (Melby et al., 1998; Strunnikov and Jessberger, 1999; Hopfner et al., 2000; Holmes and Cozzarelli, 2000; Hirano et al., 2001; Anderson et al., 2001), we observe indirect tethering of DNA molecules via multiple interactions of coiled-coil arms from complexes bound to separate DNA molecules. In accordance, recent data imply that the DNA aggregation activity of *B. subtilis* SMC is likely to require interaction be-

tween coiled-coil domains (Hirano et al., 2001). It seems likely that the individual interactions between R/M and DNA and between the R/M arms are relatively weak. The R/M DNA complexes we observe by SFM (at low protein concentrations) are not stable in gel mobility shift assays, and we do not observe DNA tethering unless many R/M molecules are bound to each DNA molecule. Because the hRad50 arms are very flexible, their interactions could be dynamic. Thus, we propose that two DNA molecules are kept in contact through the net positive effect of association and dissociation of multiple hRad50 arms (Figure 4D). In addition, the flexibility of the individual arms would allow multiple arms to interact without forcing their bound DNA molecules into a rigid conformation relative to each other. In vivo, during nonhomologous end-joining, the R/M complex could provide a tether between the two ends of the same sister chromatid. During homologous recombination it could, in addition, provide a tether between a broken end and the intact sister chromatid (see also Cromie and Leach, 2001). The flexible connections between DNA molecules would allow a variety of orientations of the DNA molecules and access for more tenaciously binding factors with specific functions in DSB repair. We believe this type of connection between DNA molecules gains strength of functional diversity through the details of structural weakness.

Experimental Procedures

Scanning Force Microscopy

For SFM analysis of R/M, 20 μ l containing 400 ng of protein in protein buffer (150 mM KCl, 25 mM Tris-HCl [pH 7.8], and 10% glycerol) was deposited on freshly cleaved mica. After about 1 min, the mica was washed with water (glass distilled, SIGMA) and dried in a stream of filtered air. Samples were imaged in air at room temperature and humidity using a NanoScope IIIa (Digital Instruments), operating in tapping mode with a type E scanner. Silicon tips (Nanoprobes) were from Digital Instruments.

Images of R/M in buffer were obtained by depositing protein onto mica as described above. Without rinsing or drying, the sample was mounted onto the SFM, and 50 μ l of protein buffer was added to the liquid cell. Olympus oxide-sharpened silicon nitride tips were used for tapping mode in buffer, operated at 18 kHz.

DNA Substrates and Binding Reactions

The 1 kb fragment used for SFM experiments was obtained by a PCR reaction on M13 ssDNA template using 22 nt primers complementary to positions 5570 and 6584. The 1.8 kb nicked circular was produced as described (Ristic et al., 2001).

DNA-protein complexes for SFM were formed in 20 μ l reactions as described for gel retardation experiments (de Jager et al., 2001) without BSA. DNA and protein concentrations were as described in the legend to Figure 3. Reactions were diluted 10-fold in deposition buffer (10 mM HEPES-KOH [pH 7.5] and 10 mM $MgCl_2$), deposited, and imaged by SFM as described above.

Image Processing and Measurements

SFM images were processed only by flattening to remove background slope using NanoScope software. Measurements were done using NanoScope software. The persistence length of the hRad50 coiled coil was calculated from the measured end-to-end distance as described (Rivetti et al., 1998). We defined the end-to-end distance of the coiled coil as the distance between the end of the arm and the point at which that arm joined the central globular domain. The maximal measured end-to-end distance was used as the contour length in persistence length calculation.

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