DNA Interaction and Dimerization of Eukaryotic SMC Hinge Domains*

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The eukaryotic SMC1/SMC3 heterodimer is essential for sister chromatid cohesion and acts in DNA repair and recombination. Dimerization depends on the central hinge domain present in all SMC proteins, which is flanked at each side by extended coiled-coil regions that terminate in specific globular domains. Here we report on DNA interactions of the eukaryotic, heterodimeric SMC1/SMC3 hinge regions, using the two known isoforms, SMC1α/SMC3 and the meiotic SMC1β/SMC3. Both dimers bind DNA with a preference for double-stranded DNA and DNA rich in potential secondary structures. Both dimers form large protein-DNA networks and promote reannealing of complementary DNA strands. DNA binding but not dimerization depends on approximately 20 amino acids of transitional sequence into the coiled-coil region. Replacement of three highly conserved glycine residues, thought to be required for dimerization, in one of the two hinge domains still allows formation of a stable dimer, but if two hinge domains are mutated dimerization fails. Single-mutant dimers bind DNA, but hinge monomers do not. Together, we show that eukaryotic hinge dimerization does not require conserved glycines in both hinge domains, that only the transition into the coiled-coil region rather than the entire coiled-coil region is necessary for DNA binding, and that dimerization is required but not sufficient for DNA binding of the eukaryotic hinge heterodimer.

Eukaryotic SMC (structural maintenance of chromosomes) proteins are essential for sister chromatid cohesion and proper chromosome condensation and also act in response to DNA damage, in DNA recombination and repair, and in gene dosage compensation (for recent reviews see Refs. 1–6). SMC proteins, which are between 120 and 160 kDa in molecular mass, feature a design reminiscent of motor proteins. The C-terminal and N-terminal globular domains are separated by extended coiled-coil regions and a hinge domain in the central. Six members of the eukaryotic SMC protein family are known, named SMC1–SMC6 for most eukaryotic organisms. They form three types of heterodimers: SMC1/SMC3, SMC2/SMC4, and SMC5/SMC6. One heterodimer each constitutes a core component of a larger multiprotein complex that acts in one of the key functions described for SMC proteins. The best described complexes are cohesins (7–9), which is based on SMC1/SMC3, and condensin with its SMC2/SMC4 core (10, 11). The SMC5/SMC6 heterodimer forms a multiprotein complex that acts in postreplicative recombinational repair of DNA damage (12, 13). Also, there are several other complexes such as a mammalian complex called RC-1, which has been implicated in DNA recombination and repair reactions (14, 15), but a gene dosage compensation complex in Caenorhabditis elegans that contains SMC2 and SMC4 homologs and is responsible for DNA reannealing of complementary DNA strands into homologous DNA. Overall, these two SMC1 isoforms are approximately 50% identical in amino acid sequence and differ at their C terminus where SMC1β possesses an extension. The hinge domain amino acid sequences are 61% identical. Both SMC1 isoforms heterodimerize with SMC3.

Key to all well described functions of SMC proteins is their interaction with DNA. The large SMC complexes show distinct DNA modulating activities. The holocomplex condensin introduces positive supercoil into relaxed circular DNA in an ATP-dependent, topoisomerase I-dependent reaction (20, 21). In the presence of topoisomerase II nicked circular DNA is converted into knotted structures, and an ordered, global writhing is introduced in the DNA (20, 22). Based on imaging of the ATP-dependent supercoil reaction of condensin, a model was suggested in which the globular head domains of condensin wrap around approximately 190 bp of DNA and thereby create two compensatory supercoils at a remote site (23). Thus, condensin has the potential to intramolecularly fold DNA and thereby to contribute to chromosome condensation in metaphase. Cohesin aggregates DNA and promotes topoisomerase II-dependent intermolecular cationation of circular DNA substrates (24). Cohesin has been suggested to embrace DNA, i.e. two newly synthesized sister chromatids, in a ring-like structure, thereby ensuring sister chromatid cohesion (25, 26). The ring is formed largely by the SMC1/SMC3 dimer and is closed by two non-SMC proteins that interact with the terminal domains of the SMC proteins. The points of contact, if any, between the cohesin ring and the DNA are not known. The possibility exists that the cohesin ring interacts with the sister chromatids mainly topologically rather than by protein-DNA binding. However, SMC proteins are known to bind DNA, at least in vitro. Individual C-terminal but not N-terminal domains from a variety of yeast and higher eukaryotic SMC proteins, and one coiled-coil region from mammalian SMC3, were found to bind DNA, preferentially double-stranded (ds) substrates that contain palindromic sequences.

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The abbreviations used are: ds, double-stranded; ss, single-stranded; EMSA, electrophoresis mobility shift assay(s); TR, transitional region.
potentially form stem-loop structures, or are AT-rich (27, 28). Chromatin immunoprecipitation experiments showed preferential association of cohesins with centromeric and AT-rich regions (29–31) but do not directly reveal protein-DNA interactions. Also, the purified SMC1/SMC3 heterodimer possesses DNA-stimulated ATPase activity and reanneals complementary single strands of DNA (15).

More recently, DNA binding was reported for a homodimeric prokaryotic SMC protein fragment that contains the hinge domain and almost the entire coiled-coil regions. Deleting the coiled-coil region from the molecule rendered the hinge inactive in electrophoresis mobility shift assays (EMSA) (32). The hinge domain is thought to lend flexibility to the two-armed structure of SMC molecules and is characterized by four highly conserved glycine residues that are typical of flexible regions in a protein and may disrupt a-helical structures (33).

The consensus sequence is GXGXXG for eukaryotic proteins and GXGXXG for prokaryotes. Mutating the C-terminal four of the five glycines abolished the ability of the Bacillus subtilis SMC protein to form a homodimer and to bind DNA (32, 33).

The importance of the hinge domain for SMC dimerization was also demonstrated in an analysis of the crystal structure of a prokaryotic hinge region and of interactions of hinge regions of Saccharomyces cerevisiae Smc1p and Smc3p (25). Individual S. cerevisiae Smc1p/Smc3p heterodimer, overexpressed in insect cells, showed predominantly the V shape. Differently tagged S. cerevisiae Smc1p or Smc3p hinge domain mutants were used in pull-down assays to study their dimerization behavior. This study revealed that for dimerization no hinge region can be replaced by another and that the hinge regions cannot be replaced by a short, unrelated peptide (25). Structural analysis of the Thermotoga maritima hinge homodimer showed that it acquires a ring or doughnut-shaped structure with the N and C termini all facing one side (Ref. 25 and Fig. 1). Thus, the adjacent coiled-coil regions of one hinge region enter and leave the doughnut structure on the same side. This restrains the flexibility of the SMC dimer arms, making it more likely for the dimeric molecule to adopt a V-shaped structure rather than a completely opened, rod-like structure. V-shaped structures were most frequently observed in electron microscopy experiments (25, 34, 35). The T. maritima hinge dimer has two interaction domains on each monomer (25). These domains are formed by short b-sheets and several a-helices, two of which are involved in dimer interactions (Fig. 1). Thus, two interaction surfaces exist between the two half-rings that form the doughnut. Because the conserved glycines are in close proximity to the amino acids that directly interact, it is thought that mutating these glycines disrupts the structure of the interaction surface. In a homodimer, this would affect both interaction surfaces, and the ring would fall apart, as shown for the B. subtilis SMC (33). For the eukaryotic heterodimer it is unclear whether both interaction surfaces are required (Fig. 1).

Given the differences between prokaryotic and eukaryotic SMC dimers, notably the heterodimerization found in eukaryotes, a number of questions remain to be answered: (i) Do eukaryotic heterodimers need to dimerize for DNA interaction as seen for the prokaryotic homodimers? (ii) Are intact coiled-coil regions required for dimerization and for DNA binding of the hinge dimer? (iii) Are the highly conserved Gly residues in the eukaryotic hinge regions of the same functional importance as in their prokaryotic homodimeric counterparts? (iv) Are two intact interaction surfaces required for stable dimerization? (v) Do the hinge dimers display specificity in DNA binding, and are they active in DNA single-stranded reannealing?

This study aims at addressing these questions. In addition, characterization of DNA interactions of hinge dimers reveals that stable dimerization requires only one intact hinge-hinge interface and that not the coiled-coil region perse but rather a short transitional region into the coiled-coil region is required for hinge dimerization and DNA binding.

**EXPERIMENTAL PROCEDURES**

Cloning and Expression of Hinge Proteins—The wild-type SMC1 hinge domain contains amino acids 481–674 of bovine SMC1 (accession number AAD13141). The corresponding DNA fragment was amplified by PCR using a cdna clone (36) with primer 1 (5’-AAAA ATT CCA TAT AGA ACA ACT AGG AGA TGC C-3’) and primer 2 (5’-AAA AAG CGG GCG CTC ACA ACT TGT CTA CTA CTG G-3’). In codons 2–4 the nucleotide in the third position was changed from G to A. Primer 2 introduces a stop codon in the end of the hinge domain coding sequence. The fragment was digested with restriction endonucleases NdeI and NotI and inserted between the NdeI and NotI sites of the SMC1 hinge domain, pET-21a (+) (Novagen). The resulting plasmid was digested with NotI, and the ends were filled in with Klenow polymerase and religated. This procedure places the His6 tag encoded by pET-21a (+) out of the SMC1 hinge protein open reading frame to avoid any possible expression of a tagged peptide by the suppression of the stop codon. To express a tagged version of the SMC1 hinge domain, pET-29a (+) (Novagen Inc.) was modified by insertion of a double-stranded oligonucleotide, which codes His6 tag followed by a stop codon. The double-stranded oligonucleotide was generated by annealing two single-stranded oligonucleotides, 5’-GAT CCC ATC ACC ATC ACC ATT CAG C-3’ and 5’-GGG CGC TCA ATG ATG ATG ATG ATG G-3’. The oligonucleotide was inserted between the BamHI and NotI sites of pET-29a (+) to generate pET-29-GSHis6. A DNA sequence encoding SMC1 hinge was amplified by PCR using primer 1 and primer 3: 5’-AGC TGG ATC CGGCCA ACT TGT CTA CTA CTG TTG TC-3’. The fragment was digested with NdeI and BamHI and inserted between the NdeI and BamHI sites of pET-29-GSHis6. In the encoded peptide the SMC1 hinge domain is separated from the His6 tag by a flexible linker, Gly-Gly-Ser.

The SMC1 hinge domain lacking the TR contains amino acids 501–659 of bovine SMC1. The corresponding DNA sequence was amplified by PCR using primer 4 (5’-AAAA AAA AAA ACA TAT GGA AAG CAT CAA GCG CCT G-3’) and primer 5 (5’-AAA AAG CGG GCG CTC ACA GGT CAC TGG CCC-3’), which introduces a stop codon. The fragment was inserted into pET-21a (+) by the same procedure as used for the wild-type SMC1 hinge protein. In the SMC1GGG hinge mutant, the glycine residues in positions 540, 554, and 655 of bovine SMC1 were replaced by alanine. To introduce the mutations into the SMC1 cdna, two overlapping fragments were amplified by PCR, fragment 1 with the primers P1 (5’-GGG CCT GCA GTA TGC TGT TGG C-3’) and P2Mut (5’-AGC GAG AGA GAT CAC GTC TAT CTA CGG CCT GCT CCT G-3’) and fragment 2 with the primers P3Mut (5’-GAC GTG ATC TCT GGC CCT GCT GGC AGT GAC C-3’) and P4 (5’-GGT CTC TTA GTC GTC ACT CTG TG-3’). Fragments 1 and 2 were purified, mixed together, and used to amplify by PCR a mutagenized fragment using the primers P1 and P4. The fragment was digested with SanD1 and BglI and used to replace the corresponding SanD1-BglI fragment of the bovine SMC1 cdNA. The mutagenized clone was used to amplify and clone the sequence encoding
the hinge domain using the same procedure as for the wild-type SMC1 hinge.

Wild-type SMC3 hinge domain contains amino acids 484–690 of bovine SMC3 (accession number AAD13142) preceded by Met and followed by a Gly-Ser linker and a His6 tag. The DNA fragment was amplified using SMC3 cDNA clone (36) with primer 6 (5' /H11032 -AAA AAA CAT ATG GCT GCT AAA AGA GAA-3' /H11032 ) and primer 7 (5' /H11032 -AGC TGG ATC CGC CAC CAA GTT CTT CTT CCG-3' /H11032 ). SMC3 TR/H11002 protein contains amino acids 504–669 of bovine SMC3 preceded by Met and followed by a Gly-Gly-Ser linker and a His6 tag. The DNA fragment was amplified using primer 8 (5' /H11032 -AAA AAA AAC ATA TGG CCA TTT TAA ATG GAA TAG-3' /H11032 ) and primer 9 (5' /H11032 -AGCTGGATCCGCCATAATAGC-CTCCAGTC-3' /H11032 ). The fragments encoding the wild-type or SMC3 TR/H11002 hinge protein were inserted into the pET-29-GSHis6 by the same procedure as used for the His-tagged SMC1 hinge plasmid. In the SMC3GGG mutant, alanine was substituted for glycine in bovine SMC3 positions 662, 666, and 667. To introduce the mutations the fragment encoding the wild-type SMC3 hinge inserted into pET-29-GSHis6 was used as a template. Two overlapping fragments were amplified by PCR, fragment 3 using primer P5 (5' /H11032 -GAG GTA CAA TCC CAG ATT TGA C-3' ) and primer P6Mut (5'-AGC GGC AGT CAA AGC GGC TCG ATG AC-3') and fragment 4 using primer P7Mut (5'-CGT CTT TGA CTT CCG CTT ATT ATG ATA-3') and primer 7. Fragments 3 and 4 were purified, mixed together, and used to amplify by PCR a mutagenized fragment using the primers P5 and 7. The fragment was digested with HindIII and BamHI and used to replace the corresponding fragment in the wild-type SMC3 hinge clone.

For the expression of the wild-type SMC3 protein without a tag, a DNA fragment encoding amino acids 484–690 was amplified using primer 11 (5' /H11032 -AAA ATT GCA TAT GGA ATT CGA GAA TGC TGG-3' /H11032 ) and primer 12 (5' /H11032 -AAA AAG CGG CCG CTC AAT ATG ATA ACT CTT TC-3' /H11032 ). The fragment was inserted into pET-21a (H11001) as described for the wild-type SMC1 clone.

All of the constructs were confirmed by sequencing of the entire DNA Interaction and Dimerization of SMC Hinge Domains

Fig. 2. Purification of SMC hinge domains. A, scheme of the SMC1α and the SMC3 hinge domains. The numbers indicate amino acids. The 20-amino acid transitional region is indicated (TR), as are the three glycines mutated to alanines. B–J, purification of the SMC1α/SMC3 wild type (B), the SMC1α/SMC3GGG single mutant (C), the SMC1α/SMC3 wild type (D), the SMC1αGGG/SMC3 single mutant (E), the SMC1α hinge monomer (F), the SMC3GGG monomer from SMC1αGGG/SMC3GGG double mutant coexpression (G), the SMC3 hinge monomer (H), the SMC1αTR+/SMC3TR− dimer and SMC3TR− monomer (I), the SMC1αTR+/SMC3 dimer, and (J) the SMC1αTR−/SMC3TR− dimer. The gels were silver-stained. M, marker; L, lysate; FT, nickel column flow-through; imid., imidazole.
Incubated and rotated with 30°C presence of different NaCl concentrations, up to 1.5M, were directly bound and silver staining. The NaCl or SDS concentration at which the untagged SMC hinge domain by SDS gel electrophoresis was a 200-bp EcoRI 5' rDNA fragment excised from an array of 11 nitrogen. The viscous lysate was centrifuged (40,000 rpm, 30 min, 4°C, Ti-45 rotor), and the cleared supernatant was loaded at 4°C onto 1 ml of nickel-nitritrolactate acid resin (Qiagen Inc.). The flow-through was collected, and the resin was washed with 5 mM imidazole in buffer L, followed by stepwise elution with 20, 50, and 250 mM imidazole in buffer L. The proteins were dialyzed against 2 x 1 liter of phosphate-buffered saline supplemented with 10% glycerol, 0.05 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and the protease inhibitor mixture. The protein fractions were stored at -80°C.

Electrophoresis Mobility Shift Assay—These assays were performed as described earlier (19, 27). Briefly, 30-μl reaction mixtures containing 0.5–1 ng (3000–6000 cpm) of 32P 5'-labeled DNA in 20 mM HEPES, pH 7.5, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, and protein as indicated. After 20 min of incubation at room temperature, DNA-protein complexes were resolved by electrophoresis at 4°C in nondenaturing polyacrylamide gels in 20 mM HEPES, pH 7.5, 0.1 mM EDTA. All of the gels were fixed (60 min in 10% acetic acid, 10% trichloroacetic acid, dried, and exposed for autoradiography. Radioactively labeled DNA substrate was a 200-bp EcoRI 5' rDNA fragment excised from an array of 11 head-to-tail repeats (37). This fragment bears palindromic sequences and was found to be efficiently bound by SMC protein domains. The following DNA substrates were used as competitors: a 230-bp EcoRI-KasI M13mp18 DNA fragment (bp 6001–6231), a 232-bp AvaII fragment from pUC19 (bp 1537–2059), a 340-bp human centromeric DNA fragment from chromosome 17 (38), and full-length ss or ds M13mp18 DNA.

Reannealing and Protein-DNA Network Formation Assays—Complementary DNA strands used in the assay were obtained by heat denaturation of the 32P 5'-labeled 200-bp dsDNA fragment described above as gel shift assay substrate. The reaction mixtures (30 μl) contained 20 mM Tris·HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 0.13 μM (nucleotides) heat-denatured 200-bp 32P DNA fragment and various amounts of protein. After incubation at 37°C for 15 min, the reactions were stopped by addition of 0.1 volume of 10% SDS, 100 μg/ml proteinase K, incubated for 5 min at 37°C, and analyzed by electrophoresis in 5% polyacrylamide, 0.2% bis-acrylamide gels and autoradiography.

The assays for network formation was done as described for RecA(39) and mammalian DNA-binding proteins (19, 40). The assay measures coaggregation of DNA into DNA-protein complexes that sediment rapidly. The DNA substrate was the 32P 5'-labeled 200-bp rDNA fragment. Various amounts of protein were incubated in a volume of 50 μl with the DNA (2.5 fmol) for 20 min at room temperature in the DNA binding buffer also used for gel shift experiments. The reaction mixture was then centrifuged for 3 min at 14,000 x g. The supernatant was transferred into a scintillation vial, and the pellet was solubilized in 100 μl of 0.1% SDS and also transferred into a scintillation vial. The assay was done in triplicates, and supernatant (nonaggregated) and pellet (aggregated) DNA were measured.

DNA Mobility Assay—In one procedure, the purified hinge dimer was incubated and rotated with 30 μl of nickel resin in a low salt buffer (50 mM NaCl, 50 mM sodium phosphate, pH 8) for 1 h at 4°C. The beads were washed with the same buffer and then incubated for 30 min with stepwise increasing NaCl or increasing SDS in the same buffer. Supernatants were taken at each step, and the beads were washed at the same NaCl or SDS concentration between steps. The samples were then analyzed for the presence of the untagged SMC hinge domain by SDS gel electrophoresis and silver staining. The NaCl or SDS concentration at which the untagged domain dissociated from the bead-bound His-tagged partner was taken as a comparative indicator for stability of the various dimer proteins. Alternatively, hinge dimers present in E. coli lysates made in the presence of different NaCl concentrations, up to 1.5 x, were directly bound to nickel beads, the beads were washed with the same buffer, and the bound material was eluted with imidazole (250 mM) and analyzed as above. The results were the same for both procedures.

RESULTS

Purification and Dimerization of SMC1/SMC3 Hinge Proteins—Hinge monomers or dimers were purified from E. coli by nickel resin chromatography, followed by dialysis. All of the dimers contained only one monomer tagged with His6, the SMC3 monomer (Fig. 2A), which was generally expressed at lower levels than the SMC1 partner. In control experiments, the tag was present on the SMC1 hinge domain with the same experimental results. No monomers were found in dimer preparations, and the stoichiometry in a dimer, based on Coomassie or silver staining, was 1:1. All wild-type or mutant hinge dimers were soluble under native lysis conditions (Fig. 2). For wild-type dimers, the SMC1α/SMC3 (1α/3) and the SMC1β/SMC3 (1β/3) hinge domain dimers were purified (Fig. 2, B and D). Three glycines that correspond to the four glycines shown to be required for dimerization in B. subtilis SMC (32) were mutated in two constructs by alanine substitution as indicated in Fig. 2A. Dimeric molecules that contain one mutated monomer (1αGGG/3 or 1αGGG, indicating the three glycines mutated) still formed dimers that could be purified under the same conditions as wild type (Fig. 2, C and F). If two mutants were coexpressed (1αGGG/3GGG), no dimer was found, and only the monomeric 3GGG was bound to the nickel resin, although the 1αGGG protein was strongly expressed and present in the native lysate (Fig. 2G). Thus, one intact interaction domain is required for dimerization. The hinge domains contain approximately 20 amino acids at each terminus that represent the transition into the adjacent coiled-coil regions (TR; Fig. 2A). With increasing distance from the hinge domain, the probability of coiled-coil formation, as determined by the COILS v. 2.1 program, increases. For example, in the SMC1α hinge domain, the probability to acquire a coiled-coil structure, derived from the 21-residue window of the program, is at 0.99 for the eight distal amino acids, continuously drops to 0.35 until the hinge region starts, and decreases within 11 amino acids to 0. Calculations based on the 14- or 28-residue windows yield very similar results. Every TR contains at least seven amino acids that are calculated to have a high probability to form coiled coils, i.e. at least one heptad. Two hinge domain constructs (1αTR3 and 3TR1) lack the transitional region amino acids but still form a dimer under standard lysis and purification conditions (Fig. 2J). Combining one domain with the TR and one domain without also yielded a dimer (Fig. 2D). We also expressed the wild-type or 1αTR3 or 3TR1 construct with His6 tags on both proteins (not shown). Monomeric hinge domains, with or without the TR, were also purified individually (Fig. 2, F and H; and not shown). The monomeric hinge domains, however, were generally less soluble, with approximately 20% soluble under native conditions.

The SMC1/SMC3 Hinge Dimers Bind DNA—The purified
SMC1α/SMC3 or SMC1β/SMC3 dimeric proteins were used in series of EMSA (Figs. 3 and 4). For a standard DNA substrate, we used 0.3 ng (2.3 fmol) of a 200-bp rDNA fragment that contains repetitive sequences and has the potential to form secondary structures (stem-loops) (37). Both dimers bind DNA and at the appropriate amounts (approximately 40 ng (1.1 pmol) for SMC1α/SMC3 and 7 ng (0.2 pmol) for SMC1β/SMC3) retain the 200-bp substrate entirely near the wells at the top of the gel. This indicates formation of large protein-DNA complexes. An intermediate band of relatively high molecular weight was observed in most experiments using lower amounts (5 ng (0.14 pmol)) of SMC1α/SMC3 and was also shifted toward the loading zone at higher concentrations of the dimer. 100% binding, defined as absence of any free substrate without necessarily shifting all of the DNA into the well area, was observed at 10 ng (0.28 pmol) for SMC1α/SMC3 and 2 ng (0.06 pmol) for SMC1β/SMC3. Throughout our experiments and with several independent preparations, we noted an approximately 4-fold higher affinity to DNA of the SMC1β/SMC3 dimer compared with the SMC1α/SMC3 dimer.

The addition of salt (100 mM NaCl) completely inhibited the gel shift, but up to 10 mM MgCl2 had no effect. Minimal dsDNA substrate length was determined by incubation of either dimer with palindromic, double-stranded oligonucleotides of 65, 40, 25, 18, and 12 bp in length. Binding was observed with substrates of 25 bp or longer but not with the 18- and 12-bp substrates, indicating that between 18 and 25 bp in length are required for binding as visualized in the EMSA (not shown).

In competition experiments, we tested preferences of the hinge dimer proteins for either ds or ssDNA or for DNA that has a very high probability of forming secondary structures such as stem-loops versus DNA that has no potential to form any such structures (Fig. 4). For both dimers, a 232-bp pUC19 plasmid DNA fragment (27, 28) that lacks repetitive or palindromic sequences serves as a comparatively weak competitor (50% competition at approximately 315 pg (2.1 fmol) of competitor DNA for SMC1α/SMC3 and little competition with up to 450 pg (2.9 fmol) of competitor for SMC1β/SMC3) (Fig. 4A). In contrast, a 230-bp M13 dsDNA fragment (27, 28) that has palindromic sequences with a potentially very high stability of stem-loop structures effectively competes (approximately 50% competition for SMC1α/SMC3 at 20 pg (0.1 fmol of competitor) and for SMC1β/SMC3 at 90 pg (0.6 fmol of competitor)) (Fig. 4B). Centromeric DNA has earlier been shown to be a preferred substrate for other domains (27), and centromeric regions have been shown by chromatin immunoprecipitation experiments to be preferentially bound by cohesin (29–31). DNA binding of the hinge dimers was also effectively competed by a 340-bp centromeric DNA fragment (38) (Fig. 4C; 50% competition with 12 pg (0.04 fmol) of competitor for SMC1α/SMC3 and with 30 pg (0.11 fmol) of competitor for SMC1β/SMC3). Double-stranded DNA competes approximately 10-fold more effectively than ssDNA.

**Fig. 4.** Gel shift competition assays with SMC1α/SMC3 (1α/3 Hi; 20 ng of protein) or SMC1β/SMC3 (1β/3 Hi; 26 ng of protein) hinge dimers as indicated. Competition of binding to the 32P-labeled 200-bp rDNA fragment (0.3 ng) with increasing amounts of pUC-232 (A), M13–230 (B), centromeric (C, Centr.-340 bp) DNA fragments, single-stranded, circular M13 DNA (D, ssM13), or double-stranded circular M13 DNA (E, dsM13).
as seen in a direct comparison of M13mp8 ssDNA with M13mp8 dsDNA (Fig. 4, m D and E).

Stable Hinge Dimerization Requires Only One Intact Interaction Surface—It has been reported for a prokaryotic hinge homodimer that four highly conserved glycines are essential for dimerization (33). Replacement of the four glycines with alanine abolished homodimerization, and substitution of two or three of the glycines weakened dimerization. There are two interaction surfaces between the hinge monomers (Fig. 1), which in a homodimer are both affected by the mutation. In eukaryotes, there are three glycines that correspond to the prokaryotic ones. However, as SMC proteins form heterodimers, each of the hinge monomers contributes its specific glycines, allowing testing of whether both or only one interaction surface is sufficient for dimerization. Dimers mutated in either one of the monomers, or in both, would also allow analysis of the effect on DNA binding, because dimerization has been shown to be required for DNA binding of a prokaryotic homodimer (32). Therefore, we generated the SMC1αGGG and the SMC3GGG hinge mutants in which the three glycines are replaced by alanine and expressed them in three combinations as either single mutants SMC1αGGG/SMC3 and SMC1α/SMC3GGG or as the double mutant SMC1αGGG/SMC3GGG.

Both single mutants were purified at the same conditions as their wild-type counterpart, indicating that dimers are formed and are reasonably stable (Fig. 2). Because disruption of one interaction site could weaken the association between the monomers, we compared the stability of the mutants with that of wild type using increasing ionic strength or detergent resistance (Fig. 5). In two approaches, either (i) the dimers were purified from lysates prepared in the presence of various concentrations of NaCl or (ii) purified, nickel bead-bound dimer was subjected to high NaCl or increasing SDS treatment. Only the tagged SMC3 hinge binds to the beads. In the first approach above, after washing and elution with imidazole in the same high NaCl buffer, the eluted material was analyzed on SDS gels, stained with Coomassie to visualize the presence of either the dimer or the SMC3 hinge only. In the second approach above, increasing NaCl or SDS concentrations may disrupt the dimer, and thus the untagged, potentially released monomer was analyzed in the supernatants. Both procedures yielded the same results. The wild-type dimer is stable up to the highest NaCl concentration used, i.e. 1.5 M. Except for minor differences, all of the dimers dissociate at SDS concentrations of 0.02% (Fig. 5B). The SMC1αGGG/3 and SMC1α/3GGG single mutants show the same stability as the wild-type dimer (Fig. 5). The dimer formed by the SMC1αTR- and SMC3TR- domains is as stable as a dimer that bears a TR only on one of the two hinge domains (data not shown). Thus, dimer stability is very similar in wild type, the GGG single mutant, and the TR-less hinge dimers. As noted above, the GGG double mutants fail to form a dimer under any condition tested (low or high salt, inclusion of sucrose or nonionic detergents).

DNA Binding of Hinge Dimer Variants—A prokaryotic hinge homodimer domain that lacks any potential coiled-coil region has been reported not to bind DNA, and the prokaryotic hinge proteins containing four Gly to Ala substitutions failed to dimerize and to bind DNA (32, 33). Therefore, we also tested the hinge variants and mutants described above in EMSA (Fig. 6). Although the SMC1α/3GGG mutant appeared to bind somewhat less efficiently, both single mutant hinge dimer bind DNA with an affinity roughly comparable with wild type (Fig. 6, A and B). Individual TR-proteins prepared under standard salt conditions fail to bind DNA, regardless of whether the SMC1αTR- and SMC3TR- domains are both His-tagged, copurified, and tested together or whether an SMC3TR- monomer alone is tested (Fig. 6, C and D). The hinge domains lacking any TR form a dimer but do not bind DNA. As for the other proteins that lack DNA binding, there was no difference using double-stranded or single-stranded (heat-denatured) DNA (Fig. 6F). However, a dimer that contains a TR region only on one of the hinge domains (1αTR-3) binds DNA as efficiently as wild-type dimers that contain two TRs (Fig. 6E). The GGG double mutant does not form a dimer, but neither monomer binds DNA (Fig. 6, G and H). Thus, dimerization is required but not sufficient for DNA binding of the hinge domain. At least one TR, but not the entire coiled-coil region, is necessary for DNA binding.

Formation of DNA Networks and Promotion of DNA Reannealing—The EMSA indicated that hinge dimers form large protein-DNA molecules and may link DNA molecules together, creating DNA-protein networks. Network formation was therefore specifically tested in an assay that uses radioactively labeled DNA, which is incubated with the protein, the reaction mixture was subjected to centrifugation, and the radioactivity present in the supernatant or in the pellet, a precipitated DNA-protein complex, was determined (19).
We tested the SMC1/H9251/SMC3, SMC1/H9252/SMC3, and the SMC1/H9251GGG/SMC3 dimeric hinge molecules (Fig. 7). All of these dimer proteins are active in the assay, i.e., form protein-DNA networks. 50% network formation was reached with 85 ng of the SMC1/H9252/SMC3 dimer, 170 ng of the SMC1/H9251/SMC3 dimer, and 370 ng of the SMC1/H9251GGG/SMC3 dimer. This is consistent with the more efficient DNA binding seen for the SMC1/H9252/SMC3 dimer and may indicate less activity of the SMC1/H9251GGG/SMC3 mutant compared with the wild-type dimer, although the EMSA did not show differences between them.

Earlier, it has been shown that individual C-terminal domains of a variety of SMC proteins, the RC-1 complex, the SMC1β/SMC3 heterodimer, or a prokaryotic homodimer promote reannealing of complementary strands of DNA (15, 27, 28, 41, 42). Reannealing of DNA single strands is thought to reflect the potential of proteins or other agents to stabilize the double-stranded form or to help in annealing of a DNA strand to a homolog in DNA recombination. Proteins that preferentially bind dsDNA, like all eu-

FIG. 6. Gel shift assays using the hinge single mutant dimers, the TR’ dimers, or hinge domain monomers. Increasing amounts of the SMC1/H9251GGG/SMC3 (A) or SMC1α/SMC3GGG (B) single mutants, the SMC1α/SMC3 TR’ variant (C), the SMC3TR monomer (D), the SMC1α/TR’/SMC3 dimer, the SMC1α/TR’/SMC3TR’ dimer, or the SMC1α (E), or SMC3 (F) hinge monomers were incubated with 0.3 ng of the 200-bp rDNA fragment, 32P 5’-labeled. no prot., no protein.

FIG. 7. Network formation assay. Increasing amounts of the SMC1α/SMC3, the SMC1αGGG/SMC3, or the SMC1β/SMC3 hinge dimers were incubated with 1.5 pmol of 32P 5’-labeled 200-bp rDNA. After 20 min of incubation, the mixture was centrifuged, and radioactivity was measured in supernatant (noncomplexed DNA) and pellet (networked DNA).

This study aimed at deciphering features and requirements of the SMC1 and SMC3 cohesin hinge domain with respect to DNA
interaction and dimerization. Therefore, a number of eukaryotic wild-type variants, including the two SMC1 isoforms, and mutant hinge domains were expressed, analyzed for their dimerization potential, and tested in DNA interaction assays.

Generally, preparations of the SMC1β/SMC3 hinge dimer were several fold more efficient in EMSA and network assays than those of SMC1α/SMC3. The pI of the dimers is 8.08 for SMC1α/SMC3 and 8.83 for the SMC1β/SMC3, which thus is more basic and therefore may have higher affinity for DNA. However, because the fraction of dimer that is fully active in a given preparation is unknown and may vary between different proteins (it apparently did not vary much between preparations of the same protein), the relatively small differences between DNA binding efficiency of the SMC1β/SMC3 and the SMC1α/SMC3 dimers should not be overinterpreted. The same caution needs to be applied to interpreting calculations of protein:DNA ratios required for efficient binding. Nevertheless, considering the minimal binding site length of between 18 and 25 bp, up to eight protein molecules may bind to the 200-bp DNA substrate. Thus, the ratio of protein:DNA is 15:1 for SMC1α/SMC3 dimer and 3:1 for the SMC1β/SMC3 dimer/25-bp binding site (122:1 and 26:1, respectively, for the 200-bp substrate). Competition experiments show that dsDNA is a preferred binding substrate because it competes approximately 10-fold more effectively than a ssDNA substrate of the same sequence. DNA fragments with the potential to form secondary structures such as the 230-bp M13 DNA fragment or the centromeric DNA fragment are much better competitors than a fragment that lacks any such potential. These preferences are similar to those seen for the C-terminal domains (27) and likely reflect overall preferences of SMC proteins.

Dimerization turned out to require only one of the monomers to contain the three conserved glycine residues that were deemed critical for the association. Based on structural data obtained with the T. maritima homodimeric hinge molecule (25), two interaction surfaces exist between the two monomers, one at the “top” and one at the “bottom” of the ring-shaped dimer (Fig. 1). Each interaction surface contains one set of conserved glycines provided by one of the two monomers, next to a helical region and a β-sheet that both are involved in the contact between the hinge domains. Thus, in a bacterial homodimer, both interaction surfaces are affected by mutating the glycines. Not so in the eukaryotic heterodimer, where mutation of one hinge domain leaves one interaction surface intact with respect to the three glycines provided by the nonmutated partner. Therefore, the contribution to dimerization of either trio of glycines could be tested. This analysis showed that one set of glycines is sufficient to form a dimer. Surprisingly, that single mutant dimer is as stable as the wild-type dimer up to high ionic strength and as resistant to an ionic detergent, regardless whether the mutation is in the SMC1 or the SMC3 partner. The two single mutant dimers behave the same in any of the dimerization or DNA interaction assays reported here. Differences in stability between wild-type and mutant dimers revealing themselves under other conditions cannot be excluded but likely have relatively little relevance. The high stability of even the single mutant dimer may make it worthwhile to analyze the eukaryotic hinge dimer interaction surface in detail.

As expected from the prokaryotic data (33), the double mutant does not dimerize, indicating that the conserved glycines play a key role also for eukaryotic hinge dimerization. Coiled-coil regions, of which remnants were present in the T. maritima homodimer, are not required for stable dimerization of the eukaryotic heterodimer, because the TR− variant dimerizes like wild type. This behavior of the eukaryotic TR− heterodimer is similar to that of the B. subtilis hinge-only homodimer, which does not require coiled-coil regions to stably dimerize (32).

DNA binding indistinguishable from that of wild-type hinge dimers was observed for the single mutant dimers. Thus, the loss of flexibility or potential structural disturbances caused by
mutated glycines in one partner are not affecting interaction of the dimer with DNA. The SMC1αGGG/SMC3GGG double mutant fails to dimerize, and none of the monomeric hinge domains, whether bearing or lacking the TR, bind DNA. The SMC1αTR and SMC3TR domains form a dimer that nevertheless does not bind DNA. Thus, dimerization alone is not sufficient to achieve the capacity to bind DNA, but rather the TR confers this ability to the hinge dimer. This is similar to the B. subtilis hinge domain, which if lacking any coiled-coil still homodimerizes but is unable to bind DNA (32). However, it was only shown that homodimers containing a long stretch of the coiled-coil region readily interact with DNA (32). Here we demonstrate that the eukaryotic heterodimer does not require extended coiled-coil regions for DNA binding, because the TR with a few amino acids that would allow formation of just one heptad is sufficient. It remains to be determined whether an analogous short TR would confer DNA binding activity to the prokaryotic homodimer. With the help of the TR sequence, the ends of the eukaryotic hinge region may be folded and positioned correctly to enable the hinge dimer to bind DNA. It ought to be interesting to determine the exact DNA binding surface of the hinge dimer and how it is affected by the amino acids immediately adjacent to the hinge region. Could the hinge region perhaps not be directly associating with DNA, and is DNA contacted only through the TR sequences? The PI values of the four TR sequences are either slightly basic or basic; the N-terminal SMC1α TR is at 8.5, N-terminal SMC3 is at 10.0, C-terminal SMC1α is at 9.7, and C-terminal SMC3 is at 8.2. Thus, each hinge monomer contains one weakly basic and one strongly basic TR sequence, and they are juxtaposed to each other in the outgoing coiled-coil dimer rods. Our experiments, however, show that a TR on a monomeric hinge domain does not bind DNA. Thus, it is more likely that the TR induces proper folding of the hinge domains, allowing DNA binding. Future experiments should clarify the individual roles of each of these TR sequences and determine whether the one heptad and/or the basicity is critical.

The hinge domains not only bind DNA but also form large DNA-protein networks and reanneal complementary DNA strands. The reannealing capacity of other SMC domains or of complexes was seen earlier, e.g., of the mammalian RC-1 complex, its SMC1/3 dimer, a variety of C-terminal SMC domains, Schizosaccharomyces pombe condensin, or the B. subtilis SMC homodimer (15, 27, 28, 41, 42). However, in one recent study (44), the S. pombe condensin or its SMC1/3 core were reportedly not active or were weakly active in a reannealing assay, although condensin or its SMC2/4 dimer was active. It is presently unclear why this differs from the other studies. The preference of SMC proteins such as the hinge dimers to bind ds versus ssDNA may explain the reannealing feature, because the proteins may stabilize duplex regions that form at initiation of annealing and thus prevent dissociation. Network formation indicates that the hinge dimers are able to link at least two separate DNA molecules. It is not known whether one dimer links these DNA molecules or whether there is interaction between two dimers, each associated with one or more DNA molecules. Because a similar DNA reannealing activity has been seen with other domains of SMC proteins as well as with full-length dimers, it is tempting to speculate that this activity may contribute to DNA damage response-related functions of SMC complexes. For example, the activity may support the annealing of a damaged DNA strand to a single-stranded repair template.

In earlier studies, interactions between other domains of SMC proteins and DNA were described, i.e. the C-terminal domains of a variety of SMC proteins from yeast and mammals and of one coiled-coil domain of bovine SMC3 (27, 28). N-terminal domains did not bind DNA, nor did they affect binding of DNA by the C termini if included in the reaction. These studies were limited to monomeric domains. In light of the effects of dimerization seen for the hinge domains on their interaction with DNA, activities seen only on monomeric domains may not necessarily represent the activities relevant for the biologically functional unit, the SMC heterodimer. No DNA binding has been observed with a hinge-less prokaryotic homodimer (32), indicating that there are no or only minor contributions of the C-terminal and/or coiled-coil domains to DNA binding. That homodimer does not bind DNA in vitro, and the dimers, SMC head domain derived from T. maritima aggregated DNA, suggesting the potential of terminal domains, likely the C-terminal component, to interact with DNA (45). Together, contributions of the terminal domains to the interaction of a full-length eukaryotic SMC heterodimer or in the context of a larger complex can currently not be excluded. One may imagine dimerization-induced folding or activation of the ATPase located in the N terminus modulating DNA interactions of SMC proteins and thus adding to the plasticity of the system.

It remains to be determined how important direct DNA binding of SMC heterodimers is in the context of the proposed ring structure of SMC complexes (25, 26). A ring may encircle one or two DNA duplexes by binding at one or multiple sites through individual domains present in the ring, or it may primarily or even entirely encircle DNA topologically without the need for direct binding. A purely topological embrace of sister chromatids may suffice for sister chromatid cohesion. Considering the evidence for DNA binding capacity of SMC domains, including the present study, and the preferential localization of cohesin in vivo to certain chromosomal regions such as A/T-rich regions (29–31) or to sites of double-stranded breaks (46), a combination of both topological association and direct protein-DNA contact currently appears most likely.

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