Positive and negative regulation of SMC–DNA interactions by ATP and accessory proteins

Michiko Hirano and Tatsuya Hirano*

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

Structural maintenance of chromosomes (SMC) proteins are central regulators of higher-order chromosome dynamics from bacteria to humans. The *Bacillus subtilis* SMC (BsSMC) homodimer adopts a V-shaped structure with an ATP-binding catalytic domain at each end. We report here that two small proteins, ScpA and ScpB, associate with the catalytic domains of BsSMC in an ordered fashion and suppress its ATPase activity. When combined with a ‘transition state’ mutant of BsSMC that poorly hydrolyzes ATP, ScpA promotes stable engagement of two catalytic domains in an ATP-dependent manner. In solution, this occurs intramolecularly and closes the DNA-entry gate of an SMC dimer. ScpB further stabilizes this conformation and prevents BsSMC from binding to double-stranded DNA (dsDNA). In contrast, when the mutant BsSMC is first allowed to interact with dsDNA, subsequent addition of ScpA leads to assembly of large nucleoprotein complexes, possibly by stabilizing intermolecular engagement of the catalytic domains from different SMC dimers. We propose that the ATP-modulated engagement/disengagement cycle of SMC proteins plays both positive and negative roles in their dynamic interactions with dsDNA.

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Introduction

Structural maintenance of chromosomes (SMC) proteins are ubiquitous from bacteria to humans and play fundamental roles in many aspects of higher-order chromosome dynamics, ranging from chromosome segregation to dosage compensation (Hirano, 2002; Jessberger, 2002; Hagstrom and Meyer, 2003). In eukaryotic cells, two different SMC subunits form a heterodimer, which in turn associates with several non-SMC subunits to assemble a fully functional holocomplex. For example, a pair of SMC2 and SMC4 acts as the core of the condensin complex (Hirano *et al.*, 1997; Sutani *et al.*, 1999; Freeman *et al.*, 2000; Ono *et al.*, 2003), whereas SMC1 and SMC3 function as components of the cohesin complex (Losada *et al.*, 1998; Toth *et al.*, 1999; Tomonaga *et al.*, 2000;

Vass *et al.*, 2003). The two complexes possess different sets of non-SMC subunits whose functional importance has been well documented both *in vivo* (Bhat *et al.*, 1996; Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Sutani *et al.*, 1999; Lavoie *et al.*, 2002) and *in vitro* (Kimura and Hirano, 2000).

The best-studied member of the SMC family in prokaryotic organisms is the *Bacillus subtilis* SMC protein (BsSMC). Disruption of the *smc* gene causes decondensation and mis-segregation of chromosomes, indicating that the bacterial SMC protein shares related, if not identical, functions with the eukaryotic SMC complexes *in vivo* (Britton *et al.*, 1998; Graumann *et al.*, 1998; Moriya *et al.*, 1998). Although BsSMC had been thought to function as a simple homodimer (Hirano and Hirano, 1998), recent studies in genetics and bio-informatics have provided evidence that BsSMC may function together with two proteins called ScpA and ScpB (Mascarenhas *et al.*, 2002; Soppe *et al.*, 2002). This notion is further substantiated by the finding that ScpA belongs to the kleisin family of proteins that contains condensin and cohesin subunits (Schleiffer *et al.*, 2003). It remains to be determined, however, whether ScpA and ScpB indeed physically interact with BsSMC to form a stoichiometric complex and, if so, how they functionally modulate the activities of BsSMC *in vitro*.

SMC dimers adopt a very unique V-shaped structure (Melby *et al.*, 1998). A central hinge domain is flanked by two long coiled-coil arms, each having an ATP-binding cassette (ABC)-like catalytic domain in its distal end. How the mechanical action of this uniquely designed protein machine is coupled with its ATPase cycle is under active investigation (Kimura and Hirano, 1997; Kimura *et al.*, 1999; Losada and Hirano, 2001; Yoshimura *et al.*, 2002; Sakai *et al.*, 2003; Stray and Lindsley, 2003). By analogy to ABC transporters (Hopfner and Tainer, 2003), it is believed that ATP binding and hydrolysis by an SMC protein would close and open its coiled-coil arms by regulating association (engagement) and dissociation (disengagement) of the two catalytic domains, respectively. This mechano-chemical action is likely to be modulated by non-SMC subunits that bind to the catalytic domains of SMC proteins (Anderson *et al.*, 2002; Haering *et al.*, 2002). On the basis of these predictions, models have been proposed to explain how cohesin might hold two sister DNAs together (Haering and Nasmyth, 2003) or how condensin might induce superhelical tension into DNA (Swedlow and Hirano, 2003). Moreover, biochemical experiments using purified BsSMC suggest that ATP binding to the catalytic domains may promote intermolecular interactions of different SMC dimers, thereby assembling higher-order nucleoprotein complexes (Hirano *et al.*, 2001; Hirano and Hirano, 2002). Despite these accumulating lines of evidence, there has been no decisive demonstration of the ATP-modulated engagement/disengagement mechanism of SMC proteins. This is presumably due to the very transient nature of the postulated interaction between the two catalytic domains, as has been shown to be the case for ABC transporters (Moody *et al.*, 2002; Smith *et al.*, 2002).

*Corresponding author. Cold Spring Harbor Laboratory, One Bungtown Road, P.O. Box 100, Cold Spring Harbor, NY 11724, USA.
Tel.: +1 516 367 8370; Fax: +1 516 367 8815;
E-mail: hirano@cshl.org

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In the current study, we have used two strategies to better understand how the dynamic interaction of SMC proteins with DNA is modulated by ATP and accessory proteins. First, biochemical purification and reconstitution experiments show that ScpA and ScpB bind to the catalytic domains of BsSMC in an ordered fashion and suppress its ATPase activity *in vitro*. Second, a 'transition state' mutation is introduced into BsSMC so that it traps and stabilizes the ATP-dependent engagement of two catalytic domains. When the mutant protein is combined with ScpA, the mechano-chemical cycle of BsSMC is slowed down, enabling us to dissect its otherwise cryptic mode of DNA interactions. Our results suggest that ATP and the accessory proteins play both positive and negative roles in the regulation of SMC–DNA interactions.

Results

ScpA and ScpB associate with the catalytic domains of BsSMC in an ordered fashion

Three *B. subtilis* proteins, BsSMC, ScpA and ScpB, were expressed in *Escherichia coli* and purified into near-homogeneity by two-step column chromatography (Figure 1A). To test whether the three proteins physically interact with each other, we performed co-immunoprecipitation experiments. First, wild-type BsSMC was incubated with ScpA, ScpB or ScpA and ScpB together, and immunoprecipitated with an anti-BsSMC antibody. The input and bead-bound fractions were analyzed by immunoblotting with antibodies specific to BsSMC, ScpA and ScpB (Figure 1B, lanes 1–8). We found that ScpA was readily co-precipitated with BsSMC in the absence of ScpB (Figure 1B, lane 6), whereas ScpB alone bound poorly to BsSMC (Figure 1B, lane 7). When ScpA was present in the reaction mixture, however, ScpB became efficiently co-precipitated with BsSMC (Figure 1B, lane 8). Thus, ScpA apparently bridges the interaction between BsSMC and ScpB. The same results were obtained when wild-type BsSMC was replaced with a single-armed mutant (DDDD) of BsSMC (Hirano and Hirano, 2002), suggesting that the two-armed structure of BsSMC is not essential for its interaction with ScpA and ScpB (Figure 1B, lanes 9–12). Moreover, reciprocal co-immunoprecipitation experiments demonstrated that ScpA and ScpB can directly interact with each other in the absence of BsSMC (Figure 1C).

We then used various deletion mutants of BsSMC (Hirano and Hirano, 2002) to determine which part of BsSMC might interact with ScpA. The full-length or truncated BsSMC was mixed with ScpA (or buffer alone) and was subjected to immunoprecipitation with anti-ScpA. The input and bead-bound fractions were analyzed by immunoblotting with anti-BsSMC and anti-ScpA (Figure 1D). Consistent with the results shown in Figure 1B, both two-armed and single-armed BsSMC were efficiently co-precipitated with ScpA (Figure 1D, lanes 6 and 8). The head-less mutant (amino acids 160–1037), which lacked the catalytic domain, interacted only weakly with ScpA (Figure 1D, lane 10). The hinge-stalk mutant (262–861), further lacking the 'neck' region that connects the catalytic and coiled-coil stalk domains, was no longer co-precipitated with ScpA (Figure 1D, lane 12). These results suggest that the major binding site of ScpA lies in the catalytic domain of BsSMC with a possible extension to part of its neck domain.

Reconstitution of a bacterial SMC holocomplex

As judged by sucrose gradient centrifugation, the purified ScpA and ScpB proteins had sedimentation coefficients of ~ 2.6 and ~ 3.5 S, respectively (Figure 2A). Both values were consistent with the previous report that ScpA and ScpB are present as monomers and dimers, respectively (Volkov *et al.*, 2003). BsSMC dimer alone had a sedimentation coefficient of ~ 6.5 S (Figure 2B, top), as described previously (Hirano *et al.*, 2001). Next we incubated BsSMC with an excess amount of ScpA and ScpB at a molar ratio of $\sim 1:4:4$, and fractionated the mixture by sucrose gradient centrifugation (Figure 2B, bottom). Under this condition, virtually all BsSMC proteins shifted toward heavier fractions with an average sedimentation coefficient of ~ 8.6 S. Equally important, a subpopulation of ScpA and ScpB (~ 20 – 25% of input) was now co-fractionated exactly with BsSMC, indicating a successful reconstitution of a bacterial SMC holocomplex. Similarly, all BsSMC proteins shifted to the holocomplex fraction at input molar ratios of $\sim 1:2:2$ and $\sim 1:1:1$ (BsSMC:ScpA:ScpB), whereas approximately half of them remained in the dimer fraction at a ratio of $\sim 1:0.5:0.5$ (Supplementary Figure 1). These results strongly suggest that the holocomplex has a symmetrical structure with two copies each of BsSMC, ScpA and ScpB.

ScpA and ScpB modulate the DNA-binding and ATPase activities of BsSMC in distinct manners

Purified BsSMC displays higher affinity for single-stranded DNA (ssDNA) than double-stranded DNA (dsDNA) in the presence or absence of ATP as judged by gel-shift assays (Hirano and Hirano, 1998). While the mobility of ssDNA was shifted by discrete steps proportionally to the amount of input proteins, dsDNA exhibited smeared bands even at high concentrations of BsSMC (Figure 3A). Neither ScpA nor ScpB alone, nor in combination, bound to dsDNA or ssDNA, regardless of the presence or absence of ATP (Figure 3B; data not shown). We found, however, that the DNA-binding activities of BsSMC are specifically modulated in the presence of the accessory proteins. Although ScpA or ScpB alone hardly affected dsDNA binding of BsSMC (Figure 3C, top, lanes 2–4), simultaneous addition of ScpA and ScpB severely interfered with this activity (Figure 3C, top, lane 5). Similar observations were made when ATP was included in the binding reactions (Figure 3C, top, lanes 6–9). We noticed that, in the presence of ATP γ S, ScpA alone weakly suppressed dsDNA binding of BsSMC (Figure 3C, top, compare lanes 10 and 11). The effects of ScpA and ScpB on ssDNA binding of BsSMC were completely different from those on its dsDNA binding. ScpA stimulated ssDNA binding of BsSMC, producing nucleoprotein complexes larger than those formed with BsSMC alone (Figure 3C, bottom, compare lanes 2 and 3). ScpB had little effect on ssDNA binding of BsSMC either in the absence or presence of ScpA (Figure 3C, bottom, lanes 4 and 5). Inclusion of ATP in these binding reactions produced a very similar set of results (Figure 3C, bottom, lanes 6–9). ATP γ S increased the basal level of ssDNA binding by BsSMC (Figure 3C, bottom, compare lanes 2 and 10), and a possible stimulatory effect by ScpA could not be measured under this condition (Figure 3C, bottom, lane 11). These results show that ScpA, ScpB and their combination modulate the dsDNA- and ssDNA-binding activities of BsSMC in distinct manners.

ScpA or ScpB alone (or in combination) had no ATPase activity in the presence or absence of DNA (data not shown), consistent with the lack of nucleotide-binding motifs in their

amino-acid sequences. We found that ScpA suppresses both DNA-independent and DNA-stimulated ATPase activities of BsSMC in a dose-dependent manner (Figure 3D, panels 1–3). Inclusion of ScpB in the reaction mixtures further enhanced the inhibitory effect of ScpA although ScpB alone had little effect on the rate of ATP hydrolysis by BsSMC. To further characterize the inhibitory effect of ScpA, we performed a $MgCl_2$ titration experiment. ScpA greatly reduced the peak of ATPase activity observed at 0.5–1.0 mM $MgCl_2$ in the absence of DNA (Figure 3D, panel 4). It also suppressed the DNA-stimulated ATPase activity that was prominently detected at higher concentrations of $MgCl_2$ (Figure 3D, panels 5 and 6). Thus, it is most likely that ScpA affects both the intra- and intermolecular activation modes of ATP hydrolysis catalyzed by BsSMC (Hirano *et al.*, 2001).

Construction and characterization of a transition state mutant of BsSMC

Recent biochemical and structural studies of an ABC transporter (MJ0796) showed that a point mutation near the Walker B motif (E171Q) effectively traps the 'transition state' in which the two catalytic domains are stably engaged in an ATP-sandwiched manner (Moody *et al.*, 2002; Smith *et al.*, 2002). To test whether SMC proteins utilize a mechanochemical cycle analogous to that of ABC transporters, we introduced the corresponding mutation (E1118Q) into BsSMC. In the absence of ATP, the E1118Q mutant protein displayed DNA-binding activities comparable to those of the wild-type protein (Figure 4A, compare lanes 2–4 and 8–10). Remarkably, addition of ATP greatly stimulated or stabilized dsDNA binding by the E1118Q mutant (Figure 4A, lanes 11–13), but not by wild-type BsSMC (Figure 4A, lanes 5–7). ATP γ S was not as effective as ATP in stimulation of the DNA-binding activities of E1118Q (data not shown), suggesting that the mutant protein has a lower affinity for this ATP analog, as has been shown for the corresponding mutant of the ABC transporter MJ0796 (Moody *et al.*, 2002). We also found that the ability of ATP hydrolysis of the E1118Q mutant was reduced but was not completely abolished (Figure 4B).

To further understand the role of ATP in dsDNA binding by BsSMC, we used two additional mutants of BsSMC that are

Figure 1 Physical interactions among BsSMC, ScpA and ScpB. (A) Purified fractions of BsSMC, ScpA and ScpB were subjected to 10% SDS-PAGE and visualized by staining with Coomassie blue. (B) Two-armed (wild-type) BsSMC was mixed with buffer alone (lanes 1 and 5), ScpA (lanes 2 and 6), ScpB (lanes 3 and 7) or ScpA and ScpB (lanes 4 and 8), and immunoprecipitated with an antibody specific to BsSMC. In all, 10% of input (lanes 1–4) and precipitated fractions (lanes 5–8) were analyzed by immunoblotting with the indicated antibodies. The interaction of ScpA and ScpB with single-armed BsSMC was characterized in the same way (lanes 9–12). (C) Fractions containing ScpA (lanes 1, 4 and 7), ScpB (lanes 2, 5 and 8), or ScpA and ScpB (lanes 3, 6 and 9) were immunoprecipitated with anti-ScpA (lanes 4–6) or anti-ScpB (lanes 7–9). A total of 10% of input (lanes 1–3) and precipitated fractions (lanes 4–9) were analyzed by immunoblotting with the indicated antibodies. (D) ScpA was mixed with two-armed BsSMC (lanes 1 and 6), single-armed BsSMC (lanes 2 and 8), head-less BsSMC (lanes 3 and 10) or hinge-stalk BsSMC (lanes 4 and 12), and immunoprecipitated with anti-ScpA. In all, 10% of input (lanes 1–4) and precipitated fractions (lanes 5–12) were analyzed by immunoblotting with the indicated antibodies. To demonstrate the specificity of the immunoprecipitation reactions, ScpA was omitted in lanes 5, 7, 9 and 11.

Figure 2 Reconstitution of a BsSMC-ScpA-ScpB complex *in vitro*. (A) Purified ScpA (top) or ScpB (bottom) was loaded onto a 5–20% sucrose gradient and centrifuged at 42 000 rpm for 24 h in an SW50.1 rotor. Fractions were subjected to SDS-PAGE and stained with Coomassie blue. (B) Purified BsSMC (top) or a mixture of BsSMC, ScpA and ScpB (bottom) was loaded onto a 5–20% sucrose gradient and centrifuged at 45 000 rpm for 15 h in an SW50.1 rotor. Fractions were analyzed as above. The positions of protein standards (ovalbumin (3.7 S), BSA (4.6 S) and aldolase (7.3 S)) are indicated.

defective in ATP hydrolysis: the Walker A mutant K371 cannot bind to ATP, whereas the C-motif mutant S1090R does bind to ATP but is unable to promote engagement of the two catalytic domains (Hirano *et al.*, 2001). We found that the two mutants behaved almost identically to wild-type BsSMC (Figure 4C, left, compare lanes 2–3 and 6–9) under the condition where E1118Q displayed drastic ATP stimulation of its dsDNA binding (Figure 4C, left, lanes 4 and 5). The majority of the nucleoprotein complexes formed with E1118Q in the presence of ATP was readily precipitated by low-speed centrifugation in a spin-down assay (Figure 4C, right, transition, lane 13). No such DNA- and ATP-dependent precipitation was observed with wild-type BsSMC or the other two mutant proteins (Figure 4C, right). The different behavior of E1118Q and S1090R clearly indicates that ATP-dependent engagement of the catalytic domains is required for the formation of large nucleoprotein complexes and that ATP binding is not sufficient.

When the E1118Q mutation was combined with mutations that alter the hinge structure (AAAA; Hirano and Hirano, 2002), the resulting AAAA-E1118Q mutant formed precipitable complexes even in the absence of dsDNA in an ATP-dependent manner (Figure 4D, left). Sucrose gradient centrifugation of AAAA-E1118Q further confirmed the ATP-dependent formation of a large protein assembly without DNA (Figure 4D, right). The AAAA protein containing no E1118Q mutation failed to be precipitated under the same condition in both assays. These results suggest that ATP-dependent engagement of the catalytic domains can be induced intermolecularly (between different BsSMC molecules) without DNA when hinge function is compromised.

The transition state mutant of BsSMC unveils the otherwise cryptic action of ScpA

We then tested the effects of ScpA and ScpB on the dsDNA-binding activity of E1118Q mutant with two different proto-

Figure 3 Modulation of SMC activities by ScpA and ScpB. (A) A fixed concentration of dsDNA (pBluescript; 15.6 μ M nucleotides) and ssDNA ($\phi \times 174$ virion DNA; 15.6 μ M nucleotides) was incubated with increasing concentrations of wild-type BsSMC (0, 70, 140, 210, 280, 350 and 420 nM arms) in the absence of ATP (lanes 1–7). The reaction mixtures were fractionated on a 0.7% agarose gel and visualized by EtBr stain. Protein–DNA complexes are indicated by asterisks, and free DNAs are indicated by arrows. (B) dsDNA or ssDNA was incubated with no protein (lane 1), ScpA (840 nM monomers; lane 2), ScpB (840 nM monomers; lane 3) or ScpA and ScpB (840 nM each; lane 4) in the absence of ATP and analyzed as above. (C) dsDNA or ssDNA was incubated with no protein (lane 1), BsSMC alone (lanes 2, 6 and 10), BsSMC and ScpA (lanes 3, 7 and 11), BsSMC and ScpB (lanes 4, 8 and 12) or BsSMC, ScpA and ScpB (lanes 5, 9 and 13) in the absence of ATP or presence of ATP (lanes 6–9) or ATP γ S (lanes 10–13), and analyzed as above. The final concentrations of BsSMC, ScpA and ScpB used were 420, 840 and 840 nM, respectively. (D) A fixed concentration of wild-type BsSMC (300 nM arms) was mixed with increasing molar ratios of ScpA (red), ScpB (black) or ScpA and ScpB (green). The ATPase activity of the mixtures was assayed in a buffer containing 10 mM KCl and 2 mM MgCl₂ in the absence of DNA (panel 1) or in the presence of dsDNA (31.2 μ M nucleotides; panel 2) or ssDNA (31.2 μ M nucleotides; panel 3). The activity is represented as percentage of the activity of BsSMC without Scp proteins under each condition. Alternatively, the ATPase activity of wild-type BsSMC (300 nM arms) was assayed at different MgCl₂ concentrations in the absence (blue) or presence (red) of a fixed molar ratio of ScpA (SMC:ScpA 1:4; panels 4–6). The rate of ATP hydrolysis is expressed as the number of ATP molecules hydrolyzed per second per arm.

cols. In Protocol #1 (identical to that used for Figure 3C), BsSMC was first incubated with ScpA, ScpB or ScpA/ScpB in the presence or absence of ATP, and then dsDNA template was added to the protein mixtures. In the absence of ATP, the responses of E1118Q to ScpA and ScpB were virtually identical to those of wild-type BsSMC (Figure 5Aa and b,

Figure 4 Characterization of the transition state mutant of BsSMC. (A) A fixed concentration of dsDNA or ssDNA (15.6 μ M nucleotides) was incubated with no protein (lane 1) or increasing concentrations (105, 210 or 420 nM arms) of the wild-type (lanes 2–7) or E1118Q mutant (lanes 8–13) protein in the absence or presence of ATP. The reaction mixtures were analyzed as in Figure 3A–C. Protein–DNA complexes are indicated by asterisks, and free DNAs are indicated by arrows. (B) The ATPase rates of wild-type BsSMC (WT), transition state mutant (TR) and Walker A mutant (WA) were determined in a buffer containing 5 mM KCl and 2 mM MgCl₂ in the absence or presence of dsDNA or ssDNA. The rate of ATP hydrolysis is expressed as the number of ATP molecules hydrolyzed per second per arm. (C) A fixed concentration of wild-type or mutant BsSMC proteins (420 nM arms) was incubated with dsDNA in the absence or presence of ATP. The reaction mixtures were split into two aliquots, and subjected to gel-shift (lanes 2–9) and spin-down (lanes 10 and 11) assays. In the latter, the mixtures were spun at 16 000 g for 15 min, and the supernatant (sup) and pellet (ppt) fractions were analyzed by 7.5% SDS–PAGE. Lane 1 contained no protein, and lanes 10 and 11 contained no dsDNA. (D) The AAAA-E1118Q and AAAA mutants were subjected to the spin-down assay as above in the absence (left, lanes 1 and 2) or presence (left, lanes 3 and 4) of dsDNA. Alternatively, the DNA-free reactions were fractionated by 5–20% sucrose gradients and the fractions were analyzed by SDS–PAGE (right).

lanes 2–5). We found, however, that the ATP-stimulated dsDNA binding specifically observed with E1118Q was now suppressed in the presence of ScpA (Figure 5Aa, compare lanes 6 and 7). Such suppression was not observed with ScpB alone (Figure 5Aa, lane 8), but was modestly enhanced when both ScpA and ScpB were present (Figure 5Aa, lane 9). The

Figure 5 Effects of ScpA and ScpB on BsSMC mutants defective in the ATPase cycle. (A) The dsDNA-binding activities of four different BsSMC proteins (the transition state mutant E1118Q (a), wild-type BsSMC (b), the Walker A mutant K37I (c) and the C-motif mutant S1090R (d)) in the presence or absence of ScpA and/or ScpB were examined by gel-shift assays with two different protocols. In Protocol #1, BsSMC was first incubated with buffer alone (lanes 2 and 6), ScpA (lanes 3 and 7), ScpB (lanes 4 and 8) or ScpA and ScpB (lanes 5 and 9) in the absence or presence of ATP, and then dsDNA was added to the protein mixtures. In Protocol #2, dsDNA was first incubated with BsSMC alone in the absence or presence of ATP, and then supplemented with buffer alone (lanes 10 and 14), ScpA (lanes 11 and 15), ScpB (lanes 12 and 16) or ScpA and ScpB (lanes 13 and 17). The molar ratio of input proteins (SMC:ScpA:ScpB) was 1:2:2. No protein was added in lane 1. After incubation, the reaction mixtures were fractionated on 0.7% agarose gels and visualized by EtBr stain. Protein–DNA complexes are indicated by asterisks and free DNAs are indicated by arrows. (B) Interactions of four different BsSMC proteins with ScpA and ScpB were assayed by immunoprecipitation. BsSMC was incubated with ScpA and ScpB in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of ATP, and immunoprecipitated with anti-BsSMC. Each of the precipitates was split into two aliquots and washed with a buffer containing 0.1 M KCl (lanes 1 and 3) or 0.5 M KCl (lanes 2 and 4) in the continued absence or presence of ATP. Precipitated fractions were analyzed by immunoblotting with the indicated antibodies.

Walker A mutant K37I and the C-motif mutant S1090R behaved almost identically to wild-type BsSMC under these conditions (Figure 5Ac and d, lanes 2–9).

Interestingly, we obtained different results with Protocol #2, in which dsDNA was first incubated with BsSMC alone in the presence or absence of ATP and then supplemented with ScpA, ScpB or ScpA/ScpB. Without ATP, the responses of E1118Q to ScpA and ScpB were the same as those obtained with Protocol #1 (Figure 5Aa, lanes 10–13). In contrast, with ATP, addition of ScpA further stimulated or stabilized dsDNA binding of E1118Q, producing nucleoprotein complexes of a larger size (Figure 5Aa, compare lanes 14 and 15). Under this

condition, ScpB induced no additional effects either in the absence or presence of ScpA (Figure 5Aa, lanes 16 and 17). Such ScpA-dependent stimulation of dsDNA binding was not observed with any of the other BsSMC proteins (wild type, K37L, S1090R) regardless of the presence or absence of ATP (Figure 5Ab–d, lanes 10–17). These results show that ScpA regulates the interaction of BsSMC with dsDNA both negatively and positively and that it does so in an ATP-dependent manner. This action of ScpA is cryptic in wild-type BsSMC, but can readily be observed in the E1118Q mutant, suggesting that ScpA stabilizes the engaged state by further reducing the residual level of ATP hydrolysis catalyzed by E1118Q.

To rule out the possibility that ScpA and ScpB associate more stably with the E1118Q mutant than with wild-type BsSMC and the other two mutants, we compared the stability of the interactions between the different BsSMC proteins and the accessory proteins. The wild-type and three mutant proteins were incubated with a mixture of ScpA and ScpB in the presence or absence of ATP, and immunoprecipitated with anti-BsSMC. Each of the precipitates was split into two aliquots and washed with low-salt (0.1 M KCl) or high-salt (0.5 M KCl) buffer in the continued presence or absence of ATP. We found that both ScpA and ScpB were efficiently coprecipitated with all wild-type and mutant BsSMC proteins (including E1118Q) and that ATP barely affected these interactions (Figure 5B, lanes 1 and 3). While ~50% of ScpA and most of ScpB were lost after high-salt washes, little difference was observed among the four different BsSMC proteins (Figure 5B, lanes 2 and 4). Thus, the unique response of E1118Q to ScpA and ScpB is not due to its different ability to interact with the two accessory subunits.

Discussion

Physical and functional interactions of BsSMC with ScpA and ScpB

Recent studies in genetics and bioinformatics have shown that two non-SMC proteins, ScpA and ScpB, may function together with SMC protein in *B. subtilis* and in other Gram-positive bacteria (Mascarenhas *et al.*, 2002; Soppe *et al.*, 2002). The current study demonstrates that the three protein components, purified into homogeneity, indeed interact with each other, both physically and functionally, *in vitro*. We show that ScpA and ScpB bind to (or near) the catalytic domains of BsSMC in an ordered fashion, assembling a stoichiometric holocomplex with a sedimentation coefficient of ~8.6S. ScpB alone cannot stably associate with BsSMC in the absence of ScpA. This ordered assembly of the bacterial SMC holocomplex is reminiscent of that of the eukaryotic cohesin complex, in which Scc1/Rad21 apparently bridges the interaction between Scc3/SA and the SMC1–SMC3 heterodimer (Haering *et al.*, 2002; Vass *et al.*, 2003). ScpA is distantly related with Scc1 at the amino-acid sequence level (Schleiffer *et al.*, 2003), further emphasizing the architectural similarity between the prokaryotic and eukaryotic complexes. Unlike the results reported for cohesin (Arumugam *et al.*, 2003; Weitzer *et al.*, 2003), however, we find that ATP binding and hydrolysis of BsSMC have little, if any, impact on the assembly of the bacterial SMC protein complex *in vitro*.

Consistent with the current reconstitution experiment *in vitro*, previous genetic studies provided strong lines of evidence to show that BsSMC, ScpA and ScpB proteins function-

ally interact with each other *in vivo* (Lindow *et al.*, 2002; Volkov *et al.*, 2003). However, the vast majority of BsSMC is present in a homodimeric form in *B. subtilis* cell lysates as judged by immunoprecipitation and sucrose gradient fractionation (Hirano and Hirano, 1998). Our current results also show that, depending on the timing of their actions, ScpA and ScpB play either a positive or negative role in the regulation of SMC–DNA interactions. It is therefore most likely that the interactions among the three proteins *in vivo* are highly dynamic and are tightly regulated by other cellular factors and/or by DNA substrates.

Transition state mutant: a powerful tool to dissect the action of ABC ATPases

An ATP-dependent engagement mechanism for ABC transporters was proposed some time ago (Holland and Blight, 1999), but it has been substantiated only recently by the successful crystallization of various members of this class of proteins (Hopfner *et al.*, 2000; Locher *et al.*, 2002; Smith *et al.*, 2002). One of the major obstacles to progress in the field has been that the postulated engagement of the two catalytic domains is very difficult to demonstrate by biochemical means, presumably due to its very transient nature. With the notable exception of Rad50 (Hopfner *et al.*, 2000), non-hydrolyzable ATP analogs do not work or work only partially for canonical ABC transporters (Moody *et al.*, 2002) or BsSMC proteins. In the current study, we have extended our efforts to dissect the SMC ATPase cycle (Figure 6, stages 1–3) by introducing a mutation into E1118 that is predicted to stabilize the engaged state (Figure 6, stage 3). While the current data do not directly demonstrate ATP-dependent engagement of the catalytic domains of BsSMC, they strongly suggest that this mutation makes the catalytic domains ‘sticky’ in the presence of ATP and drastically alters the DNA-binding properties of the dimer *in vitro*. It is important to note, however, that the E1118Q mutant of BsSMC still retains a partial ATPase activity, whereas the equivalent mutation in an ABC transporter completely abolishes its ability to hydrolyze ATP (Moody *et al.*, 2002). E1118Q should therefore be regarded as a hypomorphic transition state mutant. We also find that the ATPase activity of BsSMC is not inhibited by vanadate (M Hirano, unpublished), an agent that effectively traps the transition state of another ABC transporter (Chen *et al.*, 2001). Thus, the nucleotide-binding pocket of SMC proteins is closely related, but not identical, to that of ABC transporters.

As judged by standard gel-shift assays, BsSMC displays a basal level of dsDNA-binding activity in the absence of ATP (Figure 6, stage 6). Addition of ATP has little impact on this reaction presumably because wild-type BsSMC rapidly hydrolyzes the bound ATP molecules. In contrast, the E1118Q mutation traps the transition state by slowing down ATP hydrolysis by BsSMC, thereby stabilizing its interaction with dsDNA (Figure 6, stages 8 and 9). This stable dsDNA binding is not observed with the C-motif mutant S1090R (Figure 6, stage 7), indicating that ATP binding to the catalytic domains is not sufficient and that their engagement is essential. The ATP-dependent engagement may occur intramolecularly within an SMC dimer to encircle DNA strands by an ‘embrace’ mechanism (Figure 6, stage 8). Alternatively, it could occur intermolecularly between different SMC dimers to gather DNA duplexes by a ‘hand-in-hand’

Figure 6 A model for positive and negative regulation of SMC-dsDNA interactions by ATP and accessory proteins. In the absence of dsDNA, BsSMC undergoes a cycle of ATP binding, engagement and ATP hydrolysis (stages 1–3). The Walker A (K371), C-motif (S1090R) or transition state (E1118Q) mutation specifically blocks each of these steps as indicated. The ATP-dependent engagement of two catalytic domains occurs only intramolecularly (stage 3) unless the hinge structure is disturbed. ScpA and ScpB associate with the catalytic domains of BsSMC in a sequential manner (stages 4 and 5). While this occurs regardless of the nucleotide-binding state of BsSMC, only one pathway starting from stage 3 is shown here for simplicity. Association of ScpA partially blocks the dsDNA-binding activity of BsSMC (stage 4), and further association of ScpB blocks it more severely (stage 5). BsSMC binds to dsDNA in an apparently cooperative manner either in the absence (stage 6) or presence (stage 7) of ATP (basal binding). When ATP-dependent engagement of the two catalytic domains is stabilized by the E1118Q mutation, stable dsDNA binding is observed (stages 8 and 9). Subsequent binding of ScpA to BsSMC further stabilizes the engaged state by inhibiting hydrolysis of the bound ATP (stages 10 and 11). The ATP-dependent engagement could occur either intramolecularly through an ‘embrace’ mechanism (stages 8 and 10) or intermolecularly through a ‘hand-in-hand’ mechanism (stages 9 and 11).

mechanism (Figure 6, stage 9). Previous evidence suggests that the hinge of BsSMC is open upon binding to DNA, thereby providing an opportunity to initiate intermolecular engagement of the catalytic domains (Hirano *et al.*, 2001). Consistently, the current study demonstrates that, when the hinge structure of the E1118Q mutant is altered by additional mutations (i.e., E1118Q-AAAA), ATP binding is able to induce formation of a large protein assembly even in the absence of DNA.

Dual roles of the accessory proteins in the regulation of SMC-DNA interactions

Our results show that ScpA alone has a significant impact on the dsDNA-binding activity of E1118Q, but not that of wild-type protein, in the presence of ATP. It is most likely that ScpA binding further stabilizes the engaged state of the mutant BsSMC by suppressing its residual level of ATPase activity. A key observation in the current work is that,

depending on the order of incubation, ScpA induces completely opposite effects on dsDNA binding by the E1118Q mutant protein. Our interpretation of these results is as follows. When the two proteins are premixed together, stable engagement of the two catalytic domains occurs within an SMC dimer and close its coiled-coil arms, thereby blocking the entry of dsDNA strands into the inter-coiled-coil space (Figure 6, stage 4). In contrast, when E1118Q is first allowed to interact with DNA, subsequent addition of ScpA stabilizes or promotes the ATP-stimulated interactions of BsSMC with dsDNA (Figure 6, stages 10 and 11). The role of ScpB in regulating SMC functions is less clear than that of ScpA because the E1118Q mutation makes little impact on the action of ScpB. Nevertheless, ScpB, in the presence of ScpA, further reduces the ATPase activity of BsSMC and prevents its initial DNA binding, supporting the idea that ScpB stabilizes the closed conformation of BsSMC in solution (Figure 6, stage 5).

The dual regulation of BsSMC by ATP and the accessory proteins reported here is tightly coupled with its intrinsic two-armed structure, which is common to all SMC proteins. We speculate therefore that a similar mechanism may also operate in the regulation of eukaryotic SMC protein complexes such as cohesin and condensin. For example, a genetic study of the cohesion factor Pds5 in fission yeast has provided evidence that it may play both positive and negative roles in the establishment and maintenance of cohesin-mediated linkage between sister chromatids (Tanaka *et al.*, 2000). By analogy to the action of ScpA reported here, we suggest that Pds5 mediates the apparently conflicting functions by a single mechanism (i.e., stabilizing engaged conformations of cohesin either before or after the establishment of cohesion). Recent genetic studies in budding yeast have also shown that mutant forms of cohesin defective in ATP hydrolysis fail to be loaded onto chromosomes *in vivo* (Arumugam *et al.*, 2003; Weitzer *et al.*, 2003), supporting the importance of the engagement/disengagement cycle of cohesin in its loading process.

Complex modes of SMC–DNA interactions

While the current study is focused on the dsDNA-binding properties of BsSMC, protein titration experiments suggest that BsSMC interacts with dsDNA and ssDNA in distinct manners. Furthermore, the effects of ScpA and ScpB on the DNA-binding activity of BsSMC are drastically different between the two substrates. It is nevertheless important to emphasize that the two-armed structure of BsSMC is essential for both dsDNA and ssDNA binding (Hirano and Hirano, 2002). One possibility is that BsSMC interacts with ssDNA independently of its engagement/disengagement cycle. For example, ssDNA, but not dsDNA, may be capable of threading through the inter-coiled-coil space of BsSMC without opening of the arms. Such a DNA conformation-dependent threading mechanism has been proposed recently based on the analysis of a eukaryotic SMC2–SMC4 heterodimer (Stray and Lindsley, 2003). The current study also provides additional evidence that the actions of the two-armed SMC proteins may be highly dynamic and plastic, possibly involving a diverse array of intramolecular and intermolecular interactions (Hirano *et al.*, 2001; Sakai *et al.*, 2003; Stray and Lindsley, 2003). Future studies will be required to draw a more comprehensive picture of SMC–DNA interactions, and to compare and contrast the prokaryotic and eukaryotic SMC protein machines.

Materials and methods

Expression and purification of BsSMC, ScpA and ScpB

Expression and purification of wild-type BsSMC and its mutant derivatives were performed as described previously (Hirano *et al.*, 2001; Hirano and Hirano, 2002). A full-length coding sequence of ScpA was amplified by a polymerase chain reaction (PCR) from *B. subtilis* genomic DNA (Hirano and Hirano, 1998), and subcloned into the *NheI* and *HindIII* sites of pET-23b (Novagen) to generate pBG105. ScpA protein (with a His₆ tag at its C-terminus) was expressed and purified from a 1-l culture of BL21(DE3)pLysS containing pBG105, as described previously for BsSMC (Hirano *et al.*, 2001; Hirano and Hirano, 2002) with some modifications. A lysate was prepared in 40 ml of lysis buffer (50 mM Na-phosphate (pH 8.0), 300 mM NaCl and 10% glycerol) and spun at 23 000 *g* for 30 min. The supernatant was mixed with a 5-ml Ni-NTA metal affinity superflow resin (Qiagen), and incubated at 4°C for 1 h. The

resin was transferred to a column (1.5 × 3.0 cm), and washed with the following: (1) 20 ml of wash-1 buffer (50 mM Na-phosphate (pH 6.0), 300 mM NaCl, 50 mM imidazole, 10% glycerol and 5 mM 2-mercaptoethanol); (2) 20 ml of wash-2 buffer (50 mM Na-phosphate (pH 7.5), 300 mM NaCl, 50 mM imidazole, 10% glycerol and 5 mM 2-mercaptoethanol); (3) 20 ml of wash-3 buffer (wash-2 buffer containing 1 M NaCl instead of 300 mM NaCl); (4) 20 ml of wash-2 buffer. The bound proteins were eluted with 20 ml of elution buffer (50 mM Na-phosphate (pH 7.5), 300 mM NaCl, 500 mM imidazole, 10% glycerol and 5 mM 2-mercaptoethanol). The peak fractions were pooled (5–10 ml) and dialyzed against buffer M (20 mM K-Hepes (pH 7.7), 1 mM EDTA and 10% glycerol) containing 50 mM KCl and 5 mM 2-mercaptoethanol. The dialysate was applied to a 1-ml Hi-TrapQ HP column (Amersham Biosciences), and eluted with a 4-ml KCl gradient (100–600 mM) in buffer M containing 5 mM 2-mercaptoethanol. The peak fractions were pooled (~0.5 ml), and dialyzed against buffer M containing 50 mM KCl and 1 mM 2-mercaptoethanol, aliquoted and stored at –70°C. The typical yield of ScpA from a 1-l culture was ~0.7 mg. To construct a plasmid (pBH103) that expresses ScpB protein (with a His₆ tag at its N-terminus), the corresponding full-length coding sequence was amplified and subcloned into the *BamHI* and *EcoRI* sites of pRSETA (Invitrogen). ScpB was expressed and purified from a 0.5-l culture of BL21(DE3)pLysS containing pBH103 as described above with minor modifications. All the procedures involving Ni-NTA metal affinity purification were scaled down to half and the third (wash-3 buffer) and fourth (wash-2 buffer) washing steps were omitted. The typical yield of ScpB from a 0.5-l culture was ~5 mg. Rabbit antibodies were raised against the purified ScpA and ScpB, and affinity-purified as described previously (Hirano *et al.*, 1997).

Co-immunoprecipitation of BsSMC, ScpA and ScpB

In Figure 1B, 1 µg of BsSMC (two-armed or single-armed) was mixed with no protein, 1 µg of ScpA, 1 µg of ScpB or 1 µg each of ScpA and ScpB in 25 µl of a reaction mixture containing 20 mM Tris–HCl (pH 7.5), 100 mM KCl, 2.5 mM MgCl₂, 0.1% Tween 20 and 0.4 mg/ml BSA. In Figure 1C or Figure 1D, BsSMC or ScpB was omitted, respectively. After incubation at 37°C for 30 min, 5 µg of anti-BsSMC (or 10 µg of either anti-ScpA or anti-ScpB) covalently coupled to 20 µl of protein A agarose beads (Invitrogen) was added. The beads were incubated on ice for 1 h, and washed four times with the same buffer. In Figure 5B, 1 µg of BsSMC (wild type or mutants) was incubated with 1 µg each of ScpA and ScpB in 25 µl of the reaction buffer in the presence or absence of 1 mM MgATP, and recovered with the anti-BsSMC beads. The beads were then split into two aliquots and washed four times with the reaction buffer (or the same buffer containing 0.5 M KCl) in the presence or absence of 1 mM MgATP. Proteins bound to the beads were analyzed by immunoblotting using appropriate antibodies.

Sucrose gradient centrifugation

A 16 µg portion of BsSMC was mixed with 16 µg each of ScpA and ScpB (or no protein) in 100 µl of a reaction mixture containing 20 mM Tris–HCl (pH 7.5), 7.5 mM KCl and 2.5 mM MgCl₂, and incubated at 37°C for 30 min. The mixture was overlaid onto a 5-ml sucrose gradient (5–20%) made in the same buffer, and spun at 45 000 rpm at 4°C for 15 h in an SW50.1 rotor (Beckman). After fractionation, the protein samples were TCA-precipitated, subjected to SDS–PAGE and stained with Coomassie blue (Figure 2B). Alternatively, a reaction mixture containing 16 µg of ScpA alone (or ScpB alone) was processed as above and spun at 42 000 rpm at 4°C for 24 h (Figure 2A).

Gel-shift, spin-down and ATPase assays

In Protocol #1 (Figures 3C and 5A, left panel), BsSMC was first incubated at 37°C for 5 min with buffer alone, ScpA, ScpB or a mixture of ScpA and ScpB in the presence or absence of 1 mM MgATP. Negatively supercoiled dsDNA (pBluescript KS[+]) was then added to the protein mixtures and incubated at 37°C for another 60 min. In Protocol #2 (Figure 5A, right panel), BsSMC alone, after preincubation at 37°C for 5 min, was mixed with the dsDNA substrate in the presence or absence of MgATP at a final concentration of 1 mM, and incubated at 37°C for 30 min. The reaction mixture was then supplemented with buffer alone, ScpA, ScpB or a mixture of ScpA and ScpB, and incubated at 37°C for

another 30 min. In both protocols, the molar ratio of input proteins (SMC:ScpA:ScpB) was 1:2:2 (420:840:840 nM), and the final reaction mixtures (10 μ l) contained 20 mM Tris-HCl (pH 7.5), 7.5 mM KCl and 2.5 mM MgCl₂. After incubation, the reaction mixtures were fractionated on 0.7% agarose gels at 2.8 V/cm and visualized by ethidium bromide (EtBr) stain. Spin-down and ATPase assays were performed as described previously (Hirano *et al.*, 2001).

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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