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ATP-dependent aggregation of single-stranded DNA by a bacterial SMC homodimer

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SMC (structural maintenance of chromosomes) proteins are putative ATPases that are highly conserved among Bacteria, Archaea and Eucarya. Eukaryotic SMC proteins are implicated in a diverse range of chromosome dynamics including chromosome condensation, dosage compensation and recombinational repair. In eukaryotes, two different SMC proteins form a heterodimer, which in turn acts as the core component of a large protein complex. Despite recent progress, no ATP-dependent activity has been found in individual SMC subunits. We report here the first biochemical characterization of a bacterial SMC protein from *Bacillus subtilis*. Unlike eukaryotic versions, the *B.subtilis* SMC protein (BsSMC) is a simple homodimer with no associated subunits. It binds preferentially to single-stranded DNA (ssDNA) and has a ssDNA-stimulated ATPase activity. In the presence of ATP, BsSMC forms large nucleoprotein aggregates in a ssDNA-specific manner. Proteolytic cleavage of BsSMC is changed upon binding to ATP and ssDNA. The energy-dependent aggregation of ssDNA might represent a primitive type of chromosome condensation that occurs during segregation of bacterial chromosomes.
Keywords: ATPase/*Bacillus subtilis*/chromosome organization/SMC proteins/ssDNA

Introduction

Chromosomal DNA undergoes dynamic structural changes associated with critical functions. Most dramatic alterations are observed during mitosis, when rod-shaped chromosomes are assembled and segregated into two daughter cells in a highly orchestrated fashion. Recent studies have revealed that SMC (structural maintenance of chromosomes) proteins are key regulators in chromosome condensation and sister chromatid cohesion in the mitotic cell cycle (Strunnikov *et al.*, 1993, 1995; Hirano and Mitchison, 1994; Saitoh *et al.*, 1994; Saka *et al.*, 1994; Guacci *et al.*, 1997; Hirano *et al.*, 1997; Michaelis *et al.*, 1997; Losada *et al.*, 1998). SMC proteins have also been shown to participate in other apparently unrelated chromosomal events such as dosage compensation and recombinational repair (Chuang *et al.*, 1994; Jessberger *et al.*, 1996; Lieb *et al.*, 1998), leading to a consensus that this family of proteins is essential for a wide variety of higher-order chromosome dynamics in eukaryotic cells (reviewed by Koshland and Strunnikov, 1996; Hirano, 1998; Jessberger *et al.*, 1998).

SMC proteins are large polypeptides (between 1000 and 1500 amino acids long) that share a common 'head–rod–tail' configuration. The N-terminal head domain contains an ATP-binding motif (the so-called Walker A motif) and the C-terminal tail domain contains a conserved sequence termed the DA-box (perhaps related to the Walker B motif). The central rod domain consists of two long coiled-coil regions connected by a conserved 'hinge' sequence. Eukaryotes have multiple SMC proteins in single organisms: for example, four SMC proteins have been found in the budding yeast *Saccharomyces cerevisiae* (Koshland and Strunnikov, 1996) and the African toad *Xenopus laevis* (Hirano and Mitchison, 1994; Losada *et al.*, 1998). Emerging lines of evidence suggest that two different SMC proteins form a heterodimer, which in turn acts as the core component of a large functional complex. For example, 13S condensin, a protein complex essential for chromosome condensation in *Xenopus* egg extracts, consists of two SMC subunits (SMC2 type and SMC4 type) and three non-SMC subunits (Hirano *et al.*, 1997). RC-1, a recombination complex purified from calf thymus, contains two SMC subunits (SMC1 type and SMC3 type) and DNA polymerase ε and ligase III (Jessberger *et al.*, 1996). The heterodimeric association of two different SMC proteins is not random: other eukaryotic SMC complexes also obey a strict 'pairing rule' of SMC2–SMC4 or SMC1–SMC3 (Sutani and Yanagida, 1997; Lieb *et al.*, 1998; Losada *et al.*, 1998).

SMC proteins are also conserved in prokaryotic species. Strunnikov *et al.* (1993) originally found sequences structurally related to SMC proteins in two bacteria, *Mycoplasma hyorhinis* (Notarnicola *et al.*, 1991) and *Rhodospirillum rubrum* (Falk and Walker, 1988). The subsequent genome projects have identified additional homologous sequences from several bacterial species including *Bacillus subtilis* (Oguro *et al.*, 1995; Kunst *et al.*, 1997), *Mycobacterium tuberculosis* (Cole *et al.*, 1998), *Mycoplasma genitalium* (Fraser *et al.*, 1995), *Mycoplasma pneumoniae* (Himmelreich *et al.*, 1996) and the cyanobacterium *Synechosystis sp.* (Kaneko *et al.*, 1996). Furthermore, the identification of SMC-like sequences in archaeal genomes has demonstrated that this class of proteins is conserved in the third branch of life (Bult *et al.* 1996; Klenk *et al.*, 1997). Each of the bacterial or archaeal genomes contains a single SMC open reading frame (ORF). It is also interesting to note that the genomes of *Escherichia coli* and *Haemophilus influenzae* have no SMC gene (Fleischmann *et al.*, 1995; Blattner *et al.*, 1997). Although the cellular functions of the bacterial and archaeal SMC genes are poorly understood, recent genetic studies in *B.subtillus* have suggested an important role of an SMC gene in chromosome organization and partitioning in bacterial cells (Britton *et al.*, 1998; Moriya *et al.*, 1998).

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Since ATP-binding motifs are conserved in all SMC proteins, it has been proposed that SMC proteins have an ATP-dependent activity that modulates DNA or chromatin structure (Strunnikov *et al.*, 1993; Hirano and Mitchison, 1994). Despite extensive efforts, no ATPase or ATP-dependent activity has been found in individual SMC proteins, although some activities were found in holocomplexes containing SMC subunits (Jessberger *et al.*, 1996; Kimura and Hirano, 1997; Sutani and Yanagida, 1997). Thus, it is not clear whether SMC proteins alone are capable of hydrolyzing ATP or what the 'intrinsic' activity is that all SMC proteins might share. In this paper, we report the first biochemical characterization of a bacterial SMC protein from *B.subtilis*. The bacterial SMC protein is a simple homodimer that exhibits an ATPase activity without any associated subunits. We find, to our surprise, that the SMC protein interacts preferentially with single-stranded DNA (ssDNA) and forms large aggregates of ssDNA in an ATP-dependent manner. We propose that the *B.subtilis* SMC-mediated aggregation of ssDNA might represent a primitive type of chromosome condensation that occurs in bacterial cells.

Results

Expression and purification of the B.subtilis SMC protein

The genome of the Gram-positive bacterium *B.subtilis* contains a single SMC gene (P51834/Z99112) (Oguro *et al.*, 1995). The conceptual ORF encodes a 1188-amino-acid polypeptide with a calculated mol. wt of 135.3 kDa. The encoded protein shares all structural motifs conserved in members of the SMC family. Homologous sequences are restricted in the head, hinge and tail domains (Figure 1A) and the three domains are connected by two long heptad repeat regions predicted to form coiled-coils (Figure 1B). In this paper, we refer to this protein as BsSMC (for *B.subtilis* SMC).

A DNA fragment containing the ORF was subcloned into an expression vector and a recombinant BsSMC was expressed in *E.coli*. The recombinant protein exhibited an apparent mol. wt of 135 kDa on SDS–PAGE, a value consistent with the predicted mol. wt. We purified the recombinant SMC protein to homogeneity by ammonium sulfate precipitation followed by five successive column chromatographies: gel filtration, heparin–Sepharose, phosphocellulose, hydroxyapatite and denatured DNA–cellulose. The final fraction was >95% pure, as judged by Coomassie Blue stain (Figure 2A, left). By sucrose gradient centrifugation and gel filtration, we measured the sedimentation coefficient and the Stoke's radius of the purified BsSMC to be 6.6S (Figure 2B, top) and ~12 nm (not shown), respectively. By combining the two hydrodynamic parameters (Siegel and Monty, 1966), the native mol. wt of BsSMC was estimated to be ~280 kDa, suggesting that it formed a simple homodimer. A non-recombinant form of BsSMC was also purified from a *B.subtilis* extract by immunoaffinity and heparin Sepharose column chromatography. The final fraction contained a single BsSMC polypeptide whose mol. wt and sedimentation coefficient were indistinguishable from those of the recombinant protein (Figure 2A, lane 9, and 2B).

BsSMC homodimer is a DNA-stimulated ATPase

The purified BsSMC exhibited a basal level of ATPase activity in the absence of DNA. At low to medium salt concentrations (5–75 mM KCl), the ATPase activity was stimulated greatly in the presence of ssDNA, and far less efficiently in the presence of double-stranded DNA (dsDNA; Figure 3A). At high salt concentrations (>100 mM KCl), no DNA stimulation was observed, whereas the basal activity remained. The ATPase activity of BsSMC displayed different sensitivity to Mg^{2+} under different salt concentrations. At 55 mM KCl, the DNA-independent ATPase activity peaked at 0.5–1.0 mM $MgCl_2$ and decreased sharply with increasing concentrations of $MgCl_2$ (Figure 3B). The ssDNA-dependent ATPase activity was most evident around 2.5 mM $MgCl_2$. At 5 mM KCl, on the other hand, the ssDNA-dependent ATPase activity remained high in a broad range from 2.5 to 10 mM $MgCl_2$ (Figure 3C). Both the DNA-dependent and DNA-independent ATPase activities cofractionated exactly with BsSMC after sucrose gradient centrifugation (data not shown). For further confirmation, we constructed a BsSMC protein with a point mutation (Lys37 to Ile37) in the Walker A motif, which was expected to impair both the binding and hydrolysis of ATP (Rao *et al.*, 1988). The purified mutant protein (Figure 2A, lane 8) also behaved as a homodimer, as judged by gel filtration and sucrose gradient centrifugation (data not shown), but completely lacked both the DNA-dependent and DNA-independent ATPase activities under the different conditions tested (Figure 3D). These results suggest that the two ATPase activities are intrinsic to the wild-type BsSMC protein.

BsSMC binds preferentially to ssDNA

The preferential stimulation of the BsSMC ATPase activity by ssDNA suggests that BsSMC may have a higher affinity for ssDNA than for dsDNA. To test this, we assayed for DNA binding properties of BsSMC by a gel-shift assay. A fixed amount of ssDNA or dsDNA was mixed with increasing concentrations of BsSMC in a buffer containing 7.5 mM KCl and 2.5 mM $MgCl_2$, and the resulting DNA–protein complexes were fractionated on an agarose gel. As expected, BsSMC bound efficiently to ssDNA in a dose-dependent manner (Figure 4A, top, lanes 1–10). In contrast, the binding of BsSMC to dsDNA was very poor under the same conditions (Figure 4A, bottom, lanes 1–10). Little difference was observed in the ssDNA-binding in the presence or absence of ATP, suggesting that the DNA-binding was independent of ATP (see Discussion). Consistent with this notion, the ATP-binding site mutant protein bound to ssDNA in an efficiency similar to the wild-type protein either in the presence or the absence of ATP (Figure 4B).

ATP-dependent aggregation of ssDNA by BsSMC

Although the ATPase activity of BsSMC was stimulated by ssDNA, the ssDNA-binding activity of BsSMC was found to be independent of ATP, as judged by the gel shift assay. To seek a functional relationship between the two activities, we set up a spin-down assay in which an ATP-dependent interaction between BsSMC and ssDNA could be detected. BsSMC was incubated with or without ssDNA in the presence or absence of ATP, and the mixture was spun by low-speed centrifugation (16 000 *g*). When

A Head

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B. subtilis          FKSFAERISVD-FVKGVTAVVGPNGSGKSNITDAIRWVLGGQSARSLRGGKMEDII   11- 65
M. tuberculosis      FKSFAAPTTLR-FEPGITAVVGPNGSGKSNVVDALAWVMGEQGAKTLRGGKMEDVI   95-149
M. hyorhinis         FKSFADPISIN-FDGSVVGIVGPNGSGKSNINDAIRWVLGEQSAKQLRGLNMDDVI   12- 66
M. genitalium        FKSYADEITID-FTHSMTGIVGPNGSGKSNVVDALKWVLGERSMKHLRSKSGDDMI   12- 66
M. pneumoniae        FKSYADEITIN-FTHSMTGIVGPNGSGKSNVVDALKWVLGERSMKHLRSKSGDDMI   12- 66
M. jannaschii        FKSF-KKLSLD-IPKGFTAIVGPNGSGKSNIVDAILFVLGKTSAKKLRANRFSGLI   12- 65
Synechocystis. sp    FKSFGGTTAIP-FLPGFTVVSGPNGSGKSNILDALLFCLGLATSKGMRAERLPDLV   12- 66
XCAP-E               FKSYAQRTEINGFDPLFNAITGLNGSGKSNILDSICFLLGISNLTQVRASNLQDLV   11- 66
XCAP-C               FKSYAGERILGPFHKRFSCIIGPNGSGKSNVIDSMLFVFGYRAQK-IRSKKLSVLI   86-140
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Hinge

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B. subtilis          VTLEGDVVNPGGSMTGG   649-665
M. tuberculosis      VTVDGDLVGA-GWVSGG   722-737
M. hyorhinis         VSLEGDLFRPGGTITGG   548-564
M. genitalium        VTLDGETVYAGGIINGG   546-562
M. pneumoniae        VTLNGETVYAGGIIQGG   546-562
M. jannaschii        VTLDGDVIEPSGAMIGG   645-661
Synechocystis. sp    VTLEGDLLEASGAMSGG   664-680
XCAP-E               VTLGGDTFDPQGTLSGG   650-666
XCAP-C               VTLQGQIIEQSGTMTGG   732-748
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Tail

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B. subtilis          LSGGERALTAIALLFSILKVRPVPFCVLDEVEAALDEANVFRFAQYLKKYSSDTQFIVIT   1091-1150
M. tuberculosis      LSGGEKALTAVAMLVAIFRARPSPFYIMDEVEAALDDVNLRRLLSLFEQLREQSQIIIIT   1187-1246
M. hyorhinis         FSGGEKAIIAISLLFAILKARPIPLCILDEVEAALDESNVIRYVEFLKLLKENTQFLIIT    878- 937
M. genitalium        LSGGEKTLVALSVLFSILKVSAFPLVILDEAESALDPANVERFANIIKTASKNTQFLIIT    880- 939
M. pneumoniae        LSGGEKTLVALSVLFSILRVSAFPLVILDEAESALDPANVERFANIIGNSSNNTQFLIIT    880- 939
M. jannaschii        MSGGXKSLTALAFLFAIQRLNPSPFYVLDEVDAALDVKNVSLIADMIKNASKDSQFIVIS   1081-1140
Synechocystis. sp    MSGGEKSLTALSFIFALQRYRPSPFYGFDEVDMFLDGANVEKLSKMVRKQAQQAQFIVVS   1111-1170
XCAP-E               LSGGQRSLVALSLILAMLLFKPAPIYILDEVDAALDLSHTQNIGQMLRTHFRHSQFIVVS   1085-1144
XCAP-C               LSGGEKTLSSLALVFALHHYKPTPLYFMDEIDAALDFKNVSIVAFYIYEQTKNAQFIIIS   1185-1244
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B

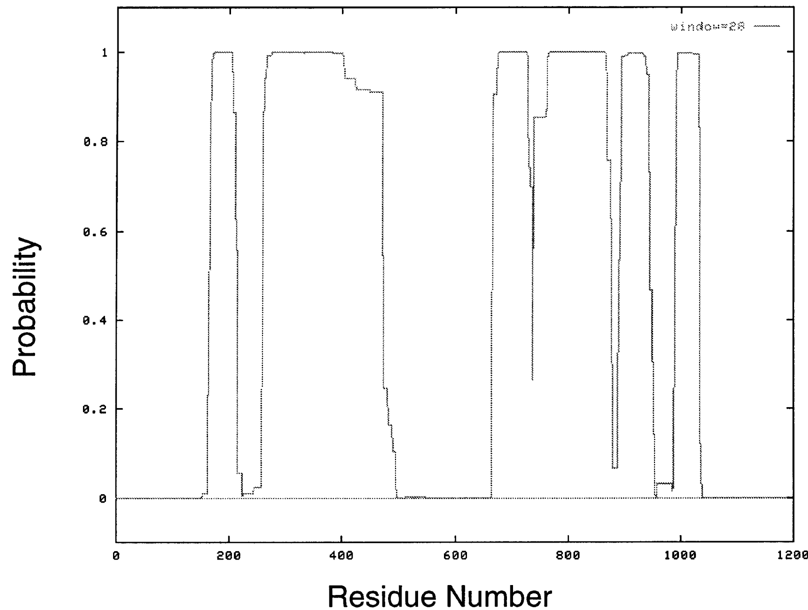

Fig. 1. Sequence analysis of BsSMC protein. (**A**) Sequence alignments of the three conserved regions (Head, Hinge and Tail). (**B**) Coiled-coil prediction of BsSMC (Lupas *et al.*, 1991).

no DNA was added, a very small amount (<5%) of input BsSMC was recovered in the pellet regardless of the presence or absence of ATP (Figure 5A, lanes 1 and 2). When ssDNA was added, however, ~70% of input BsSMC was precipitated only in the presence of ATP (Figure 5A, lanes 5 and 6). The ATP-dependent precipitation of BsSMC was ssDNA-specific: only a background level of precipitation was detected when dsDNA was incubated (Figure 5A, lanes 9 and 10). The ATP-binding site mutant protein failed to form precipitable complexes under all conditions tested (Figure 5A, lanes 3, 4, 7, 8, 11 and 12). Next, we examined the effects of ATP analogs and other nucleotides using the wild-type protein in this spin-down assay. The assembly of the precipitable complexes was readily detectable in the presence of ATP or ATPγS (Figure 5B, lanes 9 and 12), but was greatly suppressed in the presence of ADP, AMP–PNP or GTP (Figure 5B, lanes 10, 11 and 13). BsSMC had a lower affinity to AMP–PNP compared with ATP and ATPγS, as judged by photoaffinity cross-linking (data not shown), suggesting that ATP-binding,

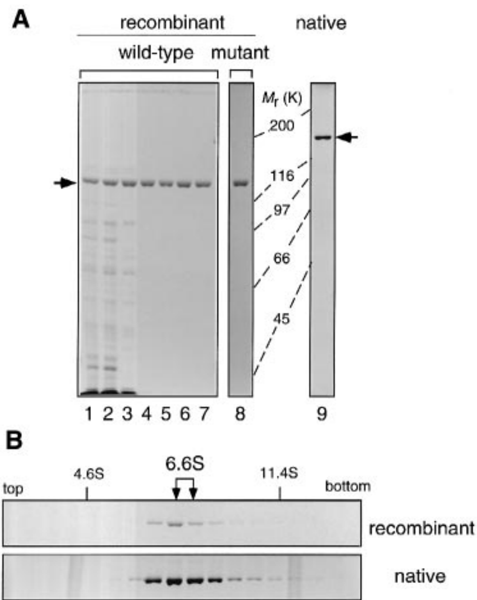

Fig. 2. Purification and characterization of BsSMC protein. (**A**, left) Purification of the recombinant *B.subtilis* SMC protein from *E.coli*. Total lysate of an overexpressing strain (lane 1); ammonium sulfate precipitates (lane 2); Sephacryl S-300 fraction (lane 3); heparin–Sepharose fraction (lane 4); phosphocellulose fraction (lane 5); hydroxyapatite fraction (lane 6); DNA–cellulose fraction (lane 7). The protein samples were fractionated by 7.5% SDS–PAGE and stained with Coomassie Blue. The ATP-binding site mutant protein was purified in the same way, and the final DNA–cellulose fraction was shown (lane 8). (**A**, right) A native (non-recombinant) form of BsSMC purified from a *B.subtilis* lysate. The native BsSMC protein was purified by immunoaffinity and heparin–Sepharose column chromatography. The final fraction was subjected to 7.5–15% linear gradient SDS–PAGE and stained with silver (lane 9). The positions of BsSMC are indicated by arrows. (**B**) Fractionation of the recombinant and native BsSMC proteins by sucrose gradient centrifugation. After fractionation, protein samples were subjected to 7.5% SDS–PAGE and stained with Coomassie Blue (top; recombinant protein) or silver (bottom; native protein).

but not hydrolysis, is important for the ssDNA aggregation reaction. The replacement of ATP with these nucleotides had little effect on ssDNA-binding, as judged by the gel-shift assay (data not shown). Little precipitation was observed in the absence of ssDNA under these conditions (Figure 5B, lanes 1–7). These results indicate that BsSMC forms large nucleoprotein aggregates specifically with ssDNA in an ATP-dependent manner.

A titration experiment revealed that the precipitation reaction was sensitive to input protein concentrations. A protein:DNA ratio of one BsSMC homodimer per 30 nucleotides was used as the standard condition (Figure 5). Increasingly higher levels of precipitation were detected at higher ratios of protein to DNA (one homodimer per 10–20 nucleotides), whereas only a background level of precipitation was observed at a ratio of one dimer per 60 nucleotides (data not shown). Salt and $MgCl_2$ concentrations also affected the precipitation reactions. For the experiments described above, 7.5 mM KCl and 2.5 mM $MgCl_2$ were used as the standard condition. The efficiency of precipitation was reduced significantly at 20 mM KCl, and abolished at 50 mM KCl, whereas ssDNA-binding of BsSMC was stable at 200 mM KCl, as judged by the gel-shift assay. Precipitation was efficient at 10 mM $MgCl_2$, but reduced greatly at 20 mM $MgCl_2$ (data not shown). These results suggest that the ATP-dependent aggregation of ssDNA is mediated by protein–protein interactions rather than by base pairing or secondary structure present in ssDNA (see Discussion).

BsSMC has an ATP-stimulated DNA-reannealing activity

DNA reannealing is an activity that promotes the conversion of two complementary ssDNAs into a double-stranded native form. Two different SMC heterodimers from eukaryotic cells have been shown to exhibit this activity, which is independent of ATP (Jessberger *et al.*, 1996; Sutani and Yanagida, 1997). To test if BsSMC has a similar activity, we mixed a fixed amount of heat-denatured linearized DNA with increasing concentrations of BsSMC in the presence or absence of ATP. After incubation, SDS was added to remove proteins and the resulting DNA was analyzed by agarose gel electrophoresis (Figure 6). In the absence of ATP, only a weak reannealing activity was detected at the highest protein concentration tested (one homodimer per 25 nucleotides; Figure 6, lanes 3–8). The reannealing activity was stimulated dramatically in the presence of ATP (Figure 6, lanes 9–14). Efficient conversion of ssDNA into dsDNA was observed at a medium concentration of BsSMC (one homodimer per 100 nucleotides; Figure 6, lane 12). At higher concentrations of BsSMC, high-molecular-weight DNA aggregates were assembled and accumulated in the well of the agarose gel (Figure 6, lanes 13 and 14, arrow). The aggregates were likely to be branched structures containing both ssDNA and dsDNA regions. ATP could not be replaced with AMP–PNP or ADP, whereas ATPγS partially stimulated the reannealing reaction. When the ATP-binding site mutant protein was used in the same assay, a weak reannealing activity was detected, but no ATP stimulation was observed (data not shown).

ATP-dependent changes in proteolytic cleavage of BsSMC

The results described above prompted us to test whether BsSMC might undergo conformational changes upon binding to ATP and ssDNA. We examined this possibility by limited proteolysis of BsSMC with trypsin. BsSMC was incubated with a buffer containing ATP or no ATP, and then ssDNA or no DNA was added. After incubation at 37°C for 30 min, trypsin was added and the reaction was terminated at time intervals. The digested proteins were analyzed by SDS–PAGE followed by silver staining. Locations of the trypsin-sensitive sites were roughly estimated by immunoblotting of duplicated samples with an antibody specific to the C-terminal sequence of BsSMC (Figure 7A). In the absence of DNA, a major cleavage occurred within the hinge domain, producing an N-terminal 78 kDa and a C-terminal 64 kDa fragment (Figure 7A, lanes 2 and 5; Figure 7B). The 78 kDa fragment was stable in the presence of ATP (Figure 7A, upper panel, lanes 6 and 7), but was unstable in its absence (Figure 7A, upper panel, lanes 3 and 4). The 64 kDa fragment was stable under both conditions. In addition, a stable 106 kDa band, a cleavage product in the first rod domain, was detected only in the presence of ATP (Figure 7A, compare lanes 4 and 7). When BsSMC bound to ssDNA, more extensive cleavages occurred regardless

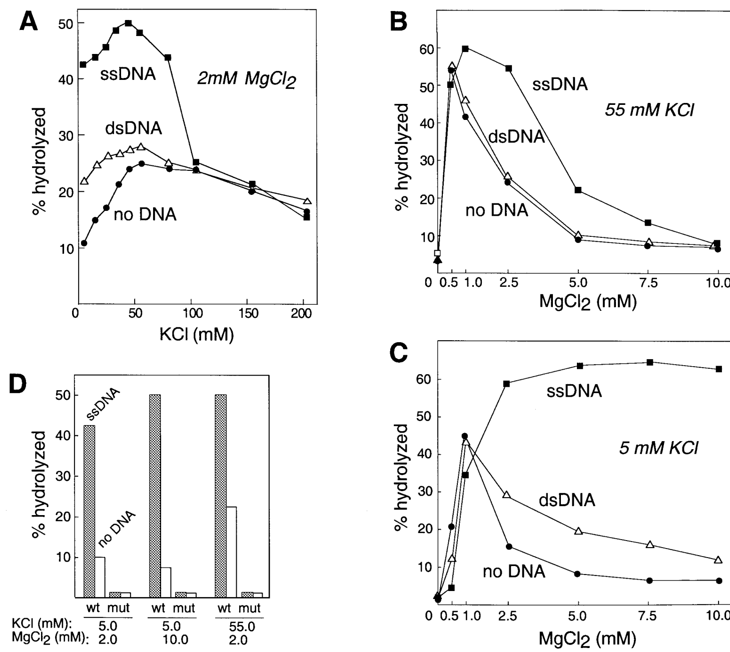

Fig. 3. The BsSMC protein is a ssDNA-stimulated ATPase. (**A**) KCl titration of ATPase activity at an $MgCl_2$ concentration of 2 mM. ATPase activity of BsSMC was assayed at different KCl concentrations in the absence of DNA, in the presence of ssDNA or in the presence of dsDNA. ATP and hydrolyzed ADP were separated by thin layer chromatography and quantitated. The activity is shown as percentage conversion of ATP to ADP. (**B**) $MgCl_2$ titration of ATPase activity at a KCl concentration of 55 mM. (**C**) $MgCl_2$ titration of ATPase activity at a KCl concentration of 5 mM. (**D**) ATPase activities of the wild-type and mutant proteins. ATPase activity was assayed under three different buffer conditions in the presence or absence of ssDNA.

of the presence or absence of ATP (Figure 7A, lanes 8–14). The stable appearance of the 106 and 78 kDa fragments was still dependent on ATP, whereas an additional 56 kDa fragment was detected specifically in the presence of both ATP and ssDNA (Figure 7A, lanes 13 and 14). Incubation with dsDNA induced no changes in the proteolytic cleavages of BsSMC. When the ATP-binding site mutant protein was used in the same assay, ATP-dependent changes of cleavage were not observed, whereas the ssDNA-induced cleavage remained intact (data not shown). In addition to trypsin, we used two other proteases with different specificity (chymotrypsin and endoproteinase Asp-N) and also detected a similar ATP-dependent response in the wild-type BsSMC protein. These results show that ATP and ssDNA can alter proteolytic cleavage patterns of BsSMC in specific and distinguishable manners.

Discussion

We report here the first biochemical analysis of an SMC protein from a non-eukaryotic organism. The *B.subtilis* SMC protein is a homodimer of two identical SMC subunits and is structurally much simpler than eukaryotic SMC complexes. The homodimeric association of identical SMC polypeptides is likely to be a common property of bacterial and archaeal SMC proteins because the bacterial and archaeal genomes fully sequenced so far contain single (or no) SMC genes. It is reasonable to speculate that the BsSMC homodimer represents the prototype of eukaryotic SMC complexes. Since this ‘primitive’ SMC protein is an active ATPase without any accessory subunits, characterization of this protein should facilitate our understanding of the intrinsic activities associated with SMC

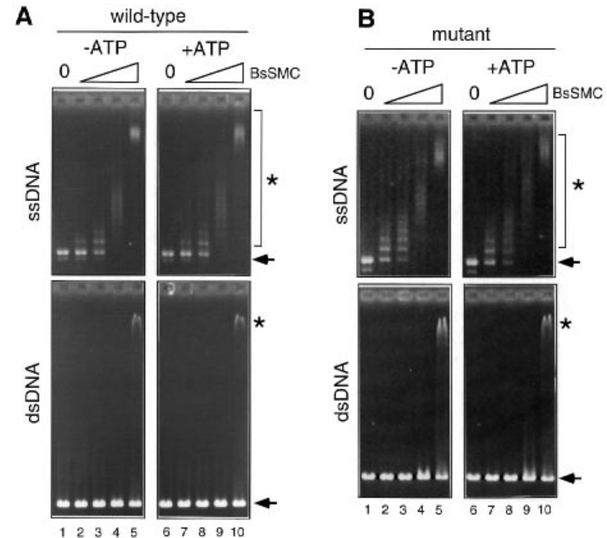

Fig. 4. The BsSMC protein binds preferentially to ssDNA. (**A**) A fixed amount of ssDNA (top) or dsDNA (bottom) was mixed with increasing concentrations of wild-type BsSMC in the absence (lanes 1–5) or presence (lanes 6–10) of ATP. After incubation in a buffer containing 7.5 mM KCl and 2.5 mM $MgCl_2$, DNA–protein complexes were fractionated on a 0.7% agarose gel and visualized by EtBr stain. The protein:DNA ratio (one BsSMC homodimer:nucleotides) was 1:240 (lanes 2 and 7), 1:120 (lanes 3 and 8), 1:60 (lanes 4 and 9), or 1:30 nucleotides (lanes 5 and 10). No protein was added (lanes 1 and 6). (**B**) The same experiment was carried out using the ATP-binding site mutant protein. The protein:DNA ratio was 1:204 (lanes 2 and 7), 1:102 (lanes 3 and 8), 1:51 (lanes 4 and 9) or 1:26 nucleotides (lanes 5 and 10). No protein was added in lanes 1 and 6. Protein–DNA complexes are indicated by asterisks and free DNAs are indicated by arrows.

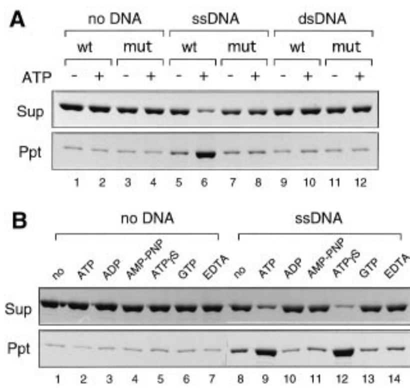

Fig. 5. ATP-dependent aggregation of BsSMC with ssDNA (containing no complementary strand). (**A**) A fixed amount of the wild-type (wt) or mutant (mut) forms of BsSMC was mixed either with no DNA, or with ssDNA or dsDNA in the absence (–) or presence (+) of ATP. After incubation in a buffer containing 7.5 mM KCl and 2.5 mM $MgCl_2$, the reactions were spun at 16 000 *g* for 15 min. Proteins recovered in the supernatants (top) and pellets (bottom) were analyzed by SDS–PAGE followed by silver staining. The protein:DNA ratio was one BsSMC homodimer per 30 nucleotides. (**B**) Effects of ATP analogs in the precipitation assay. A fixed amount of wild-type BsSMC was mixed with (ssDNA) or without (no DNA) ssDNA in the presence of ATP analogs or nucleotides indicated. Proteins recovered in the supernatants and pellets were analyzed as for (A). The protein:DNA ratio was one BsSMC homodimer per 30 nucleotides.

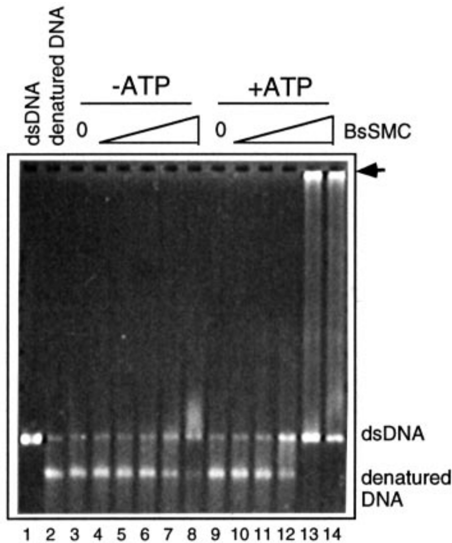

Fig. 6. ATP-stimulated reannealing of two complementary DNA strands. Heat-denatured DNA was incubated with increasing concentrations of BsSMC in the absence (lanes 3–8) or presence (lanes 9–14) of ATP. After incubation, SDS was added to remove proteins. The resulting DNA was fractionated on a 0.7% agarose gel and visualized by EtBr staining. The protein:ssDNA ratios (one BsSMC homodimer:nucleotides) were as follows: no protein (lanes 3 and 9); 1:400 (lanes 4 and 10); 1:200 (lanes 5 and 11); 1:100 (lanes 6 and 12); 1:50 (lanes 7 and 13); and 1:25 (lanes 8 and 14). Non-denatured dsDNA (lane 1) and denatured DNA used for the assay (lane 2) were also shown. dsDNA-containing large aggregates are shown by the arrow.

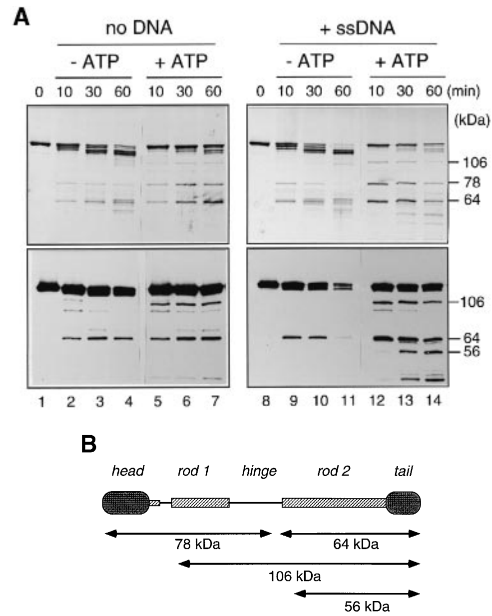

Fig. 7. ATP-dependent changes in proteolytic cleavage of BsSMC. (**A**) BsSMC was incubated with a buffer containing ATP (lanes 5–7 and 12–14) or no ATP (lanes 1–4 and 8–11), and then ssDNA (lanes 8–14) or no DNA (lanes 1–7) was added. After incubation at 37°C for 30 min, trypsin was added and the reaction was terminated at the time points indicated. The digested proteins were fractionated by 7.5% SDS–PAGE and analyzed by silver staining (top) or by immunoblotting (bottom). (**B**) Schematic presentation of the major cleavage sites. The 78 and 106 kDa fragments were stable in the presence of ATP. The 56 kDa fragment was detected in the presence of both ssDNA and ATP.

protein itself and provide fundamental insights into the basic mechanisms of SMC function.

Dynamic interactions between BsSMC and ssDNA

In this paper, we provide several lines of independent evidence that BsSMC interacts preferentially with ssDNA. The ATPase activity of BsSMC is stimulated by ssDNA more efficiently than by dsDNA under the different conditions tested. BsSMC has a high affinity to ssDNA compared with dsDNA, as judged by a gel-shift assay. The formation and the stability of the protein–ssDNA complex were not apparently affected in the presence or absence of ATP. However, this result has to be interpreted with caution. In the gel-shift assay, protein–DNA complexes were fractionated on an agarose gel in the presence of an Mg^{2+}-chelator EDTA and in the absence of ATP. This assay may not reflect the actual binding condition in solution. Indeed, spin-down and reannealing assays revealed an ATP-dependent mode of BsSMC–ssDNA interactions. In the presence of ATP, BsSMC promoted the formation of large nucleoprotein aggregates in a ssDNA-specific manner. The aggregate were formed in the absence of complementary DNA strands, and dissolved upon removal of proteins. When two complementary ssDNAs were used as substrates, they were converted into large aggregates containing dsDNA regions that were not resolved even after dissociation of BsSMC (DNA reannealing). Finally, limited proteolysis experiments suggested that BsSMC might undergo conformational changes upon binding to ATP and ssDNA. In brief, BsSMC became more resistant to proteolytic cleavage in the presence of ATP and became more sensitive when bound to ssDNA. One of the most prominent cleavages was detected in the hinge region, but it occurred independently of ATP or ssDNA. Detailed mapping of cleavage sites should provide more structural information of BsSMC in the future. Our current results, taken all together, reveal dynamic

interactions between BsSMC and ssDNA *in vitro*, and further imply that the primary target of BsSMC *in vivo* may be ssDNA (see below). Furthermore, it should be emphasized that the two activities of BsSMC reported here (ssDNA aggregation and reannealing) are the first ATP-dependent activities found associated with individual SMC proteins free of associated proteins.

Comparison with eukaryotic SMC protein complexes

Currently, only limited and fragmentary information is available on the biochemical activities associated with eukaryotic SMC complexes. Nevertheless, it is important to compare them with the activities of BsSMC reported here. Two eukaryotic SMC protein complexes have been shown to hydrolyze ATP. 13S condensin has an ATPase activity that is stimulated five-fold by dsDNA and less efficiently by ssDNA (Kimura and Hirano, 1997). RC-1 also has an ATPase activity that is only weakly stimulated by ssDNA or dsDNA (Jessberger *et al.*, 1996; R.Jessberger, personal communication). ATP hydrolysis has been detected only in the whole RC-1 complex, but not in SMC heterodimer dissociated from RC-1, leading to a proposal that the SMC heterodimer may become competent to hydrolyze ATP only after it has been assembled into functional protein complexes with non-SMC subunits (Jessberger *et al.*, 1998). In striking contrast, we found that the *B.subtilis* SMC can hydrolyze ATP in its homodimer form without any associated polypeptides. Another important difference is that, unlike the eukaryotic SMC complexes, the ATPase activity of BsSMC is stimulated by ssDNA more efficiently than by dsDNA under a wide range of different conditions tested.

The *Xenopus* 13S condensin complex introduces positive supercoils into DNA in the presence of ATP and topoisomerase I (Kimura and Hirano, 1997). We have been unable to detect a similar activity in our BsSMC fractions under different conditions tested (M.Hirano, unpublished results). Given the different properties of ATPase activities of BsSMC and 13S condensin and the preferential binding of BsSMC to ssDNA, this finding may not be surprising. We suggest that the positive supercoiling activity might be unique to a subclass of eukaryotic SMC protein complexes.

DNA reannealing activity has been found in two different SMC heterodimers from eukaryotic cells: the bovine SMC1–SMC3 heterodimer (Jessberger *et al.*, 1996) and the fission yeast cut3–cut14 (SMC4–SMC2) complex (Sutani and Yanagida, 1997). Importantly, the two eukaryotic complexes promote DNA reannealing independently of ATP, whereas BsSMC's reannealing activity is stimulated greatly in the presence of ATP. Neither SMC1–SMC3 nor cut3–cut14 has been tested for an ability to induce aggregation of ssDNA with no complementary strands.

Comparison with other DNA-interacting proteins

Are the biochemical activities of BsSMC related to those of non-SMC proteins? DNA helicases represent a large family of ssDNA-dependent ATPases that unwind DNA duplexes by using the energy of ATP hydrolysis (reviewed by Matson and Kaiser-Rogers, 1990). BsSMC shares common ATP-binding motifs (the Walker A and B motifs) with helicases and has a ssDNA-stimulated ATPase activity. However, a standard oligonucleotide displacement assay detected little, if any, helicase activity associated with BsSMC (M.Hirano, unpublished results), and the primary structure of BsSMC lacks other structural motifs conserved in all DNA helicases. It seems unlikely therefore that the ATPase activity of BsSMC is utilized to unwind the double-stranded DNA helix.

The recombination protein recA represents another group of ATPases whose activity is stimulated by ssDNA. recA has an ATP-stimulated DNA reannealing activity similar to those of BsSMC (Weinstock *et al.*, 1979). It should be noted that, although a large number of DNA-binding proteins are known to support DNA reannealing, ATP-dependent reannealing is unique to BsSMC and recA (for review of reannealing proteins, see Kowalczykowski and Eggleston, 1994). recA also promotes aggregation of ssDNA with no complementary strands, but this reaction does not require ATP (Tsang *et al.*, 1985). The recA-mediated aggregation requires a high concentration of Mg^{2+} and is relatively resistant to salt, suggesting that secondary structure present in ssDNAs may be involved in the formation of large nucleoprotein assemblies. In contrast, aggregation of ssDNA promoted by BsSMC is sensitive to salt and Mg^{2+}, and is, most importantly, ATP-dependent. It is likely that this reaction is supported by ATP-induced protein–protein interactions of BsSMC (see below).

How does BsSMC work?

We still do not know how BsSMC interacts with ssDNA and promotes its aggregation and reannealing in an ATP-dependent manner. It also remains to be determined whether the two ATP-dependent reactions are mediated by a common underlying mechanism. One possible scenario is that ATP promotes or modulates the interaction of BsSMC molecules that are bound to different single strands, thereby leading to the formation of large nucleoprotein aggregates. If two complementary strands are present in the aggregates, reannealing occurs as a result of an increased effective concentration of ssDNA molecules. The ATP-dependent, ssDNA-induced protein–protein interactions may be supported by two long coiled-coil regions of BsSMC. It will be of great interest to visualize the BsSMC–ssDNA complex by electron microscopy.

Is BsSMC involved in bacterial chromosome condensation?

Little is known about the biological functions of SMC proteins in bacterial or archaeal cells. Very recently, two groups have reported the first genetic studies of the *B.subtilis smc* gene (Britton *et al.*, 1998; Moriya *et al.*, 1998). Null mutations in *smc* cause multiple phenotypes, including accumulation of anucleate cells, disruption of nucleoid structure and misassembly of a partitioning protein complex. It has been proposed that BsSMC contributes directly to the compaction of the nucleoid mass and that defects in condensation result in the multiple phenotypes in *smc* mutants. If this model is correct, our current finding that BsSMC interacts preferentially with ssDNA would be somewhat surprising and unexpected. We hypothesize that the energy-dependent aggregation of ssDNA might represent a primitive type of chromosome

condensation that occurs during partitioning of bacterial chromosomes. Unlike eukaryotic chromosomes, bacterial nucleoids contain unconstrained negative supercoils that tend to be easily unpaired (Pettijohn, 1982). Conceivably, the energy-dependent, ssDNA-specific 'condensation' activity brings such unpaired regions together and promotes the compaction of chromosomes, thereby protecting the genetic integrity during chromosome partitioning. Alternatively, multiple and specific regions of ssDNA on bacterial chromosomes might act as a site for catalyzing chromosome condensation. In fact, Smc-GFP fusion protein localizes to discrete foci in the *B.subtilis* nucleoid (Britton *et al.*, 1998). It is interesting to note that scaffold attachment regions (SARs), *cis*-acting DNA sequences implicated in eukaryotic chromosome condensation, are AT-rich and known to be unpaired easily (Bode *et al.*, 1992).

SMC-mediated condensation might also be required for pairing of newly replicated origin regions (Lin and Grossman, 1998). It is tempting to speculate that condensation and pairing (or cohesion) of sister chromosomes are mechanistically linked and have a common evolutionary origin. In bacterial cells, the two processes are intimately related and regulated by a single SMC protein. In eukaryotes, condensation and cohesion require distinct and more functionally sophisticated SMC protein machines, condensins and cohesins (Hirano *et al.*, 1997; Losada *et al.*, 1998). We anticipate that further characterization of SMC proteins will uncover the basic mechanism underlying chromosome dynamics, which might be conserved from bacteria to humans.

Materials and methods

Cloning of the B.subtilis SMC (BsSMC) gene

Genomic DNA of *B.subtilis* was isolated as described (Munakata *et al.*, 1994). Two primers, BS7 (5′-GCGGTACCATATGTTCCTCAAACGTTTAGACG-3′) and BS6 (5′-GAGGATCCAATCCTCATCCACTTTACGG-3′), were used to amplify the BsSMC gene (P51834/Z99112) with Vent DNA polymerase (New England Biolabs). The amplified fragment was cloned into the *Nde*I and *Bam*HI sites of pRSETA (Invitrogen), and the whole sequence was determined to check that no error has been introduced during the PCR. The resulting expression plasmid was named pSO104.

Preparation of antibodies

Rabbit polyclonal antisera were raised against a synthetic peptide corresponding to the C-terminal amino acid sequence of BsSMC (KVISVKLEETKEFVQ). Immunization and affinity purification of antibodies were done as described previously (Hirano *et al.*, 1997).

Expression and purification of the BsSMC protein

A 1 l culture of BL21(DE3)pLysS containing pSO104 was grown at 37°C in Laemmli buffer broth with antibiotics, and expression of BsSMC was induced by the addition of IPTG to a final concentration of 50 μM at 30°C for 2 h. Cells were harvested, resuspended in 40 ml of lysis buffer (50 mM Na-phosphate pH 8.0, 300 mM NaCl, 1 mM PMSF), and lysed by incubation with 0.5 mg/ml lysozyme followed by sonication. 2-mercaptoethanol was then added at a final concentration of 5 mM. After a spin at 23 000 *g* for 30 min, ammonium sulfate was added to the supernatant to a final concentration of 70%. Precipitates were recovered, suspended into 18 ml of the lysis buffer containing 5 mM 2-mercaptoethanol and loaded onto a Sephacryl S-300 gel filtration column (Pharmacia; 2.5×100 cm). Fractions containing BsSMC were pooled, loaded onto a heparin Sepharose CL-6B column (Pharmacia; 1.0×6.3 cm) and fractionated with 0.1–0.6 M KCl gradient in buffer M (20 mM K-HEPES pH 7.7, 1 mM EDTA, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM PMSF). Peak fractions were pooled, dialyzed against buffer I (20 mM K-phosphate pH 7.4, 1 mM EDTA, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM PMSF) containing 50 mM KCl and applied to a P-11 phosphocellulose column (Whatman; 1.0×5.0 cm). Proteins were eluted with 0.1–0.5 M KCl gradient in buffer I, and peak fractions were pooled and dialyzed against buffer II (50 mM K-phosphate pH 7.4, 1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol, 0.1 mM PMSF). The dialysate was applied to a hydroxyapatite column (Bio-Rad; 1.0×6.3 cm), and fractionated with 50–600 mM K-phosphate gradient containing 1 mM EDTA, 10% glycerol, 5 mM 2-mercaptoethanol and 0.1 mM PMSF. The eluates were dialyzed against buffer M containing 50 mM KCl, loaded onto a denatured DNA–cellulose column (Pharmacia; 1.0×2.0 cm) and eluted with 50–600 mM KCl gradient in buffer M. The peak fractions were pooled (~3 ml), dialyzed against buffer M plus 50 mM KCl (containing 1 mM instead of 5 mM 2-mercaptoethanol and no PMSF), aliquoted and stored at –70°C. The final yield of BsSMC from a 1 l culture was ~4.5 mg. BsSMC with identical activities was obtained when the last purification step (DNA cellulose) was omitted. The mutant form of BsSMC was purified in the same way. All protein concentrations are expressed in moles of dimers.

Immunoaffinity purification of native BsSMC from B.subtilis

A native (non-recombinant) form of BsSMC was purified from a *B.subtilis* lysate by immunoaffinity column chromatography (Kimura and Hirano, 1997). In brief, a cell pellet from a 100 ml culture was suspended in 1.2 ml of buffer M (20 mM K-HEPES pH 7.7, 1 mM EDTA, 10% glycerol) containing 50 mM KCl and 1 mM PMSF, and lysed by incubation with 1.0 mg/ml lysozyme, followed by sonication. The lysate was supplemented with β-mercaptoethanol at a final concentration of 5 mM and spun at 16 000 *g* for 20 min. The supernatant was taken and KCl was added to adjust a final concentration to 100 mM. Affinity-purified peptide antibody (25 μg) was coupled to 100 μl of protein A–agarose beads (Gibco-BRL), washed and mixed with the lysate. After incubation at 4°C for 1 h, the beads were poured on a column, washed with 80 column vol of buffer M containing 100 mM KCl, 10 column vol of buffer M containing 200 mM KCl and finally 10 column vol of buffer M containing 100 mM KCl. For elution, the same buffer containing the antigen peptide at a final concentration of 0.4 mg/ml peptide was used. The eluate was further fractionated by heparin–Sepharose column chromatography or by sucrose gradient centrifugation.

Sucrose density gradient centrifugation and gel filtration

Sucrose density gradient centrifugation was performed as described, with minor modifications (Hirano *et al.*, 1997). A purified fraction was overlaid on a 5 ml sucrose gradient (5–20%) and then spun at 165 000 *g* for 15 h at 4°C in a SW50.1 rotor (Beckman). Eighteen fractions were taken manually and separated by SDS–PAGE. The gels were stained with Coomassie Blue or silver. Gel filtration was done in a Sephacryl S-300 column (Pharmacia; 0.7×50 cm). The native mol. wt was calculated as described by Siegel and Monty (1966), using a partial specific volume of 0.725. The standard proteins (Stoke's radius, S value) used were thyroglobulin (8.5 nm), catalase (5.2 nm, 11.4S) and bovine serum albumin (4.6S).

ATPase assay

ATPase assay was performed as described previously (Kimura and Hirano, 1997). In brief, BsSMC (the wild-type and mutant proteins at final concentrations of 270 and 330 nM, respectively) was mixed in a buffer containing 10 mM K-HEPES pH 7.7, 5–200 mM KCl, 0–10 mM $MgCl_2$,[α-^{32}P]ATP (1 nCi), 300 μM ATP, 5 mM K-EGTA, 1 mM dithiothreitol (DTT) and 10% glycerol. After incubation at 37°C for 30 min, SDS was added at a final concentration of 0.2%. One μl of the reaction mixture was then spotted on a PEI-cellulose thin layer chromatography (TLC) plate (Selecto Scientific) and developed in 1 M HCOOH, 0.5 M LiCl. Radiolabeled ATP and ADP were quantitated with an image analyzer (BAS 2000 Fuji Photofilm). DNA-dependent ATPase activity was assayed in the presence of 31.2 μM ssDNA (ϕX174 virion DNA; New England Biolabs) or dsDNA (ϕX174 RF form or linearized pBluescript). All DNA concentrations are expressed in moles of nucleotides.

Gel-shift assay

Reaction mixtures (10 μl) contained 20 mM Tris–HCl pH 7.5, 7.5 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.1–2.0 mM MgATP and increasing concentrations of BsSMC [final concentrations from 62.5 to 500 nM (wild-type); or from 76.3 to 610 nM (mutant)]. The mixtures were prespun at 16 000 *g* for 15 min, and the supernatants were taken and

preincubated at 37°C for 5 min. Then 15.6 μM ssDNA (φX174 virion DNA) or dsDNA (φX174 RF or linearized pBluescript) was added. After incubation at 37°C for 30 min, the reactions were mixed with 1/6 vol of a loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) and fractionated on a 0.7% agarose gel in TBE buffer. DNA was visualized with ethidium bromide.

Spin down assay

Reaction mixtures (20 μl) contained 20 mM Tris–HCl pH 7.5, 7.5 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.1–2.0 mM MgATP and 500 nM BsSMC (wild-type or mutant). The mixtures were prespun at 16 000 *g* for 15 min, and the supernatants were taken and preincubated at 37°C for 5 min. Then 15.6 μM ssDNA (φX174 virion DNA) or dsDNA (φX174 RF or linearized pBluescript) was added. After incubation at 37°C for 30 min, the reactions were spun at 16 000 *g* for 15 min, and the supernatants and pellets were analyzed by SDS–PAGE followed by silver staining (Silver Stain Plus Kit, Bio-Rad).

DNA reannealing assay

Reaction mixtures (20 μl) contained 20 mM Tris–HCl pH 7.5, 7.5 mM KCl, 2.5 mM $MgCl_2$, 0 or 2.0 mM MgATP, and increasing concentrations of BsSMC (final concentrations from 25 to 400 nM). After preincubation at 37°C for 5 min, heat-denatured, linear plasmid DNA (pBluescript) was added at a final concentration of 10.5 μM nucleotides and incubated at 37°C for another 30 min. The reaction was stopped by the addition of 2 μl of 8% SDS and 100 mM EDTA. The DNA was electrophoresed on a 0.7% agarose gel and stained with ethidium bromide.

Limited proteolysis of BsSMC

Reaction mixtures (48 μl) contained 20 mM Tris–HCl pH 7.5, 7.5 mM KCl, 2.5 mM $MgCl_2$, 0 or 2 mM MgATP, and 590 nM BsSMC. After preincubation at 37°C for 5 min, 15.6 μM φX174 ssDNA or no DNA was added and incubated further at 37°C for 30 min. Trypsin (modified, sequencing grade; Boehringer Mannheim) was then added to a final concentration of 8 ng/ml. Aliquots of 12 μl were taken at 10, 30 and 60 min, and the reactions were stopped by mixing with an equal volume of SDS sample buffer. Samples were analyzed by SDS–PAGE followed by silver staining or by immunoblotting with an antibody raised against the C-terminal peptide of BsSMC.

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