Dual roles of the 11S regulatory subcomplex in condensin functions

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Condensin is a multisubunit protein complex that reconfigures DNA structure in an ATP-dependent manner *in vitro* and plays a central role in mitotic chromosome condensation in *Xenopus* egg cell-free extracts. The *Xenopus* 13S condensin complex (13SC) is composed of two subcomplexes: an 8S core subcomplex (8SC) consisting of two structural maintenance of chromosomes (SMC) subunits (XCAP-C and -E) and an 11S regulatory subcomplex (11SR) containing three non-SMC subunits (XCAP-D2, -G, and -H). We report here the biochemical and functional dissection of this chromosome condensation machinery. Although both 8SC and 13SC can bind to DNA *in vitro* and contain the SMC ATPase subunits, only 13SC is active as a DNA-stimulated ATPase and supports ATP-dependent supercoiling activity. In the cell-free extracts, 13SC is the active form that binds to chromosomes and induces their condensation. Neither 11SR nor 8SC alone is able to bind to chromatin. Our results suggest that the non-SMC subunits have dual roles in the regulation of condensin functions: one is to activate SMC ATPases and the other is to allow the holocomplex to associate with chromatin in a mitosis-specific manner.

At the onset of mitosis, entangled interphase chromatin fibers are resolved and compacted into rod-shaped structures called mitotic chromosomes. The highly organized packaging of mitotic chromosomes is an essential step that ensures the faithful segregation of genetic materials during mitosis. Despite its fundamental importance, very little is known about the molecular mechanisms underlying the dynamic changes of higher-order chromosome structure (1, 2).

Recent studies using *Xenopus* egg cell-free extracts led to the identification of a five-subunit protein complex, termed 13S condensin, that plays a central role in both assembly and maintenance of mitotic chromosome structure (3, 4). Two core subunits of 13S condensin, XCAP-C and -E, belong to a family of chromosomal ATPases, the structural maintenance of chromosomes (SMC) family (3, 5). The remaining three subunits, XCAP-D2, -G, and -H, are not related to SMC proteins (4, 6, 7) and their possible roles in condensin regulation are poorly understood. All of the five subunits of 13S condensin are highly conserved among eukaryotes (8). Genetic studies in *Schizosaccharomyces pombe* (9, 10), *Saccharomyces cerevisiae* (11–14), *Drosophila melanogaster* (15), and *Caenorhabditis elegans* (16) have shown that they are required for chromosome condensation and segregation *in vivo*.

13S condensin, when purified from *Xenopus* egg mitotic extracts, displays a DNA-stimulated ATPase activity and reconfigures DNA structure in an ATP-dependent manner. It introduces positive supercoils into relaxed circular DNA in the presence of type I topoisomerases (17) and converts nicked circular DNA into positively knotted forms in the presence of a type II topoisomerase (18). The two ATP-dependent activities are regulated by mitosis-specific phosphorylation of the non-SMC subunits and are thought to contribute directly to mitotic chromosome condensation (6, 18). It remains unknown, however, which subunits of the 13S condensin complex are responsible for these activities. Because the SMC subunits share ATP-binding motifs and bind directly to DNA (19, 20), the SMC heterodimer alone may be sufficient to support the ATP-dependent activities. The major role of the non-SMC subunits could be to repress SMC functions during interphase, and mitosis-specific phosphorylation derepresses their inhibitory effects. Alternatively, phosphorylation of the non-SMC subunits may actively modulate the SMC subunits that would otherwise be inactive as ATPases. To distinguish between the two possibilities, biochemical and functional dissection of the condensin complex is essential.

In this paper, we describe a method of sequential immunoaffinity column chromatography in which 13S condensin and its subcomplexes can be isolated from a single batch of extracts. This has allowed us to compare biochemical activities of each purified complex *in vitro*. In addition, the ability of each complex to condense chromosomes in the cell-free extracts has been tested by combinations of differential immunodepletion and add-back of purified complexes. Our results suggest that the SMC and non-SMC subunits cooperate to support ATP-dependent positive supercoiling *in vitro* and chromosome condensation in the extracts. Finally, we describe an *in vitro* assay for studying the mechanism of mitosis-specific chromosomal targeting of condensin.

Materials and Methods

Preparation of Antibodies. Rabbit polyclonal antisera raised against the C-terminal sequences of XCAP-D2 and XCAP-H (XCAP-D2-tail and XCAP-H-tail, respectively) were described previously (4, 6). Anti-XCAP-G-tail antisera were prepared against the C-terminal sequence of XCAP-G (EKTKKNLSKLLNEEAN). A phosphopeptide (ARTKQTARKpSTGGKAPRKQLC) was used to prepare an antiserum that recognizes a phosphorylated form of histone H3 at serine 10. Affinity-purification of antibodies was performed as described previously (4, 6).

Purification of Condensin and Its Subcomplexes. The holocomplex and subcomplexes of condensin were purified from *Xenopus* egg high-speed supernatants (HSS) by sequential immunoaffinity column chromatography. An affinity-purified anti-XCAP-H-tail (200 μg) was coupled to 200 μl of protein-A-agarose beads (GIBCO/BRL). The beads were incubated with 2 ml of a mitotic HSS at 4°C for 1 h, and then poured into a 2-ml column. The column was washed consecutively with 80 column volumes of XBE2-gly [10 mM K-Hepes (pH 7.7)/100 mM KCl/2 mM $MgCl_2$/0.1 mM $CaCl_2$/5 mM EGTA/10% (vol/vol) glycerol] containing 20 mM β-glycerophosphate, 10 volumes of XBE2-gly containing 400 mM KCl and 20 mM β-glycerophosphate, and 10 volumes of XBE2-gly containing 20 mM β-glycerophosphate. To

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Abbreviations: SMC, structural maintenance of chromosomes; 8SC, 8S core subcomplex; 11SR, 11S regulatory subcomplex; 13SC, 13S condensin holocomplex; I nucleosomes, interphase nucleosomes.

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elute 11SR from the column, the XCAP-H-tail peptide was added at a final concentration of 0.4 mg/ml in XBE2-gly containing 20 mM β-glycerophosphate. The 11SR-depleted extract was mixed with 50 μg of anti-XCAP-G-tail coupled to 100 μl of protein-A-agarose beads and was incubated at 4°C for 1 h. The mixture was poured into a column and washed in the same way as described above. 13SC was eluted by the addition of the XCAP-G-tail peptide at a final concentration of 0.4 mg/ml. The unbound fraction was first incubated with anti-XCAP-G-coupled protein-A-agarose beads to ensure complete depletion of 13SC, and then used to affinity-purify 8SC with anti-XCAP-C-tail (100 μg) coupled to 100 μl of protein-A-agarose beads. In some experiments, purified fractions were concentrated with Microcon 30 concentrators (Amicon). The amounts of purified complexes were determined by SDS/PAGE followed by Coomassie blue stain using a recombinant XCAP-G as a standard.

Immunodepletion and Rescue. Immunodepletion of condensin was performed as described previously (4) with minor modifications. For complete immunodepletion of condensin subunits, a mixture containing 6 μg each of affinity-purified anti-XCAP-C, -E, and -G and 12 μg of anti-XCAP-D2 was incubated with 30 μl of Affi-Prep protein-A support (Bio-Rad) for 1 h. To deplete 13SC and 11SR, a mixture of 18 μg of anti-XCAP-G and 12 μg of anti-XCAP-D2 was used. For mock depletion, 30 μg of nonimmune rabbit IgG was used. After washing the antibody-coupled beads with XBE2, 100 μl of *Xenopus* egg HSS supplemented with an ATP-regenerating system (1 mM MgATP, 10 mM creatine phosphate, and 50 μg/ml creatine kinase) was added, and incubated on a rotating wheel at 4°C for 1 h. The supernatants were recovered and incubated with a fresh batch of the same antibody-coupled beads. After 1 h of incubation, the supernatants were recovered by two rounds of brief spins and used as depleted extracts. For add-back experiments, an affinity-purified fraction containing 11SR, 13SC, or 8SC was prepared in XBE2 and supplemented with $MgCl_2$ at a final concentration of 6 mM. Six micrograms of the purified fractions was added to 12 μl of the depleted extracts.

Preparation of Immobilized DNA. Solid-phase DNA templates were prepared according to Sandltzopoulos and Becker (21). A 2.9-kb plasmid DNA (pRSETA; Invitrogen) was digested with *Eco*RI and *Pvu*II, and the *Eco*RI site was labeled with Klenow enzyme in the presence of biotin-14-dATP. Unincorporated biotinylated nucleotides and the short fragment were removed by gel filtration using a spin column. Fifty micrograms of labeled DNA was coupled to 5 mg of streptavidin-coated paramagnetic beads (M-280; Dynal, Great Neck, NY) according to the manufacturer's instructions. The coupling efficiency was about 4 μg of DNA per 1 mg beads.

Solid-Phase Nucleosome Assembly in Egg Extracts. To assemble interphase nucleosomes (I nucleosomes), 1 μg of DNA (coupled to 250 μg of beads) was incubated with 100 μl of 2-fold-diluted interphase HSS, which was supplemented with an ATP-regenerating system and 0.002% Nonidet P-40 at room temperature for 2 h on a rotating wheel. To assemble "I→M" nucleosomes, the same amount of the DNA beads was incubated first with 100 μl of 2-fold-diluted interphase HSS for 1 h, and then with 100 μl of 2-fold-diluted mitotic HSS for another 1 h. To test the efficiency of nucleosome assembly, nucleosomal DNA (corresponding to 250 ng of DNA) was digested with 3.6–36 units of micrococcal nuclease in 30 μl of XBE2 containing 10 mM $CaCl_2$ at room temperature for 10 min. DNA was purified and electrophoresed in a 1.25% agarose gel with 0.5× TBE buffer.

DNA- and Nucleosome-Binding Assay. An affinity-purified fraction of 11SR, 8SC or 13SC (0.5 pmol) was incubated with DNA-coupled (250 ng of DNA) or uncoupled paramagnetic beads in 30 μl of XBE2-gly containing 0.002% Nonidet P-40, 5 mg/ml BSA, and increasing concentrations of KCl on a rotating wheel at room temperature for 1 h. The beads were washed twice with 200 μl of the same buffer containing no BSA, and bound proteins were analyzed by immunoblotting. For nucleosome-binding assay, 0.5 pmol of 13SC was incubated with nucleosomes assembled around immobilized DNA (250 ng) in 30 μl of XBE2-gly, 0.002% Nonidet P-40, and 5 mg/ml BSA at room temperature for 1 h. After washing the beads, bound proteins were analyzed as described above.

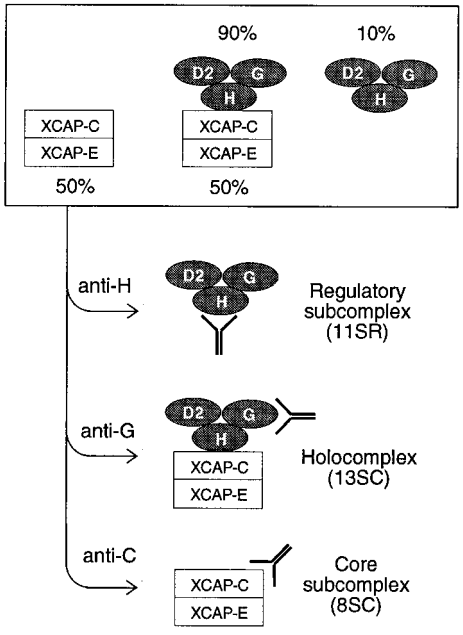

Fig. 1. A diagram for sequential affinity-purification of the holocomplex and subcomplexes of condensin. See the text for details.

Results

Purification of Holocomplex and Subcomplexes of Condensin. Our previous work showed that, in *Xenopus* egg extracts, XCAP-C and XCAP-E are present in two different complexes with sedimentation coefficients of 8 S and 13 S (4). The 8S form is a heterodimer of the two SMC subunits, whereas the 13S form contains three additional non-SMC subunits, XCAP-D2, -G, and -H. More recently, we noticed that an antibody raised against the C-terminal peptide of XCAP-H (XCAP-H-tail) immunoprecipitated a subcomplex containing XCAP-D2, -G, and -H. Neither XCAP-C nor XCAP-E was detectable in the immunoprecipitate. This was an unexpected result because a different antibody raised against a recombinant XCAP-H fragment, as well as antibodies raised against the other non-SMC subunits, immunoprecipitated all of the five subunits (refs. 4 and 17; data not shown). We reasoned that the C-terminal tail of XCAP-H is hidden inside the 13S complex but is exposed when present in the non-SMC subcomplex.

These findings allowed us to establish a sequential immunoaffinity purification scheme that yields the three different complexes in separate fractions from a single batch of extract (Fig. 1). In brief, anti-XCAP-H-tail antibody was used to purify the non-SMC subcomplex from a mitotic extract (Fig. 2*A*, lanes 1 and 4). The flowthrough fraction was subjected to an anti-XCAP-G-tail antibody column to obtain the 13S holocomplex (Fig. 2*A*, lanes 2 and 5). Finally, the 8S SMC heterodimer was purified by using anti-XCAP-C-tail antibody from the extracts

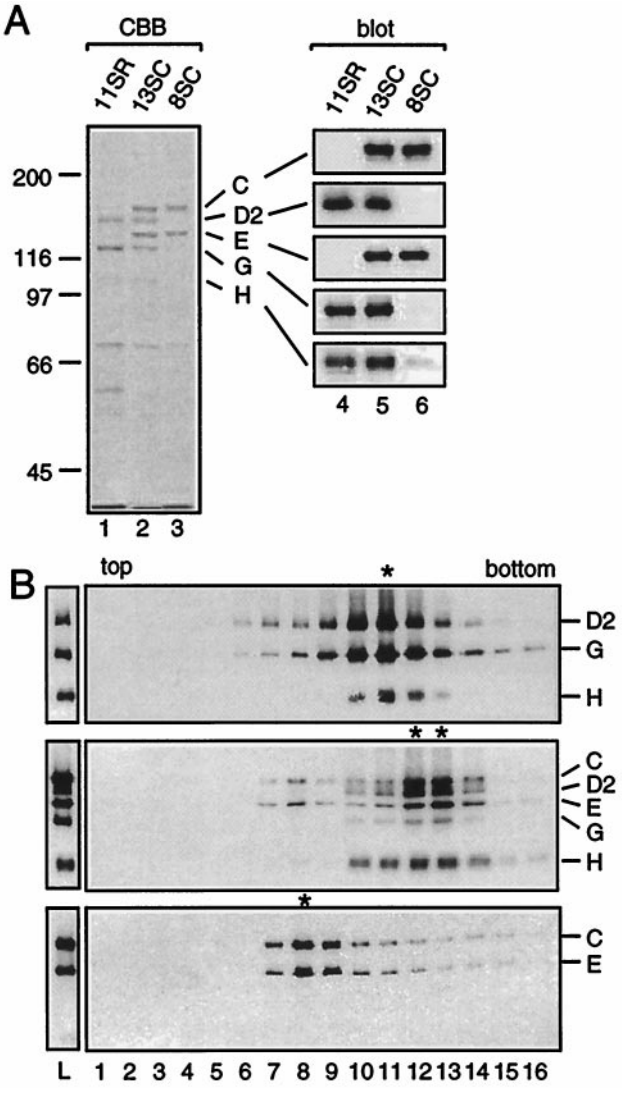

Fig. 2. Characterization of purified complexes. (*A*) Affinity-purified fractions of 11SR (lanes 1 and 4), 13SC (lanes 2 and 5), and 8SC (lanes 3 and 6) were subjected to SDS/7.5% PAGE, and stained with Coomassie blue (lanes 1–3) or analyzed by immunoblotting with antibodies against condensin subunits (lanes 4–6). (*B*) An affinity-purified fraction of 11SR (*Top*), 13SC (*Middle*), or 8SC (*Bottom*) was fractionated in a 5–20% sucrose gradient. Fractions were resolved by SDS/7.5% PAGE and analyzed by immunoblotting.

that had been depleted of the non-SMC subunits (Fig. 2*A*, lanes 3 and 6). When the purified non-SMC subcomplex was subject to sucrose gradient centrifugation, XCAP-D2, -G, and -H co-fractionated in a single peak of 11S, confirming their tight association (Fig. 2*B*, *Top*). In this manuscript, we refer to this subcomplex as 11SR (for 11S regulatory subcomplex). An affinity-purified holocomplex containing XCAP-C, -E, -D2, -G, and -H sedimented in a 13S peak (Fig. 2*B*, *Middle*), whereas the SMC heterodimer displayed an 8S peak (Fig. 2*B*, *Bottom*) as described previously (4). For simplicity, we refer to the 13S condensin holocomplex as 13SC and the 8S core subcomplex as 8SC. As judged by quantitative immunoblotting, about 50% of the SMC subunits exist in 8SC and 50% in 13SC (4), whereas about 90% of the non-SMC subunits exist in 13SC and 10% in 11SR (data not shown). The low abundance of 11SR explains why this population of the non-SMC subunits was overlooked before. Given the current results, it is most likely that the "13S condensin" fraction previously obtained with an anti-XCAP-G affinity column (6, 17, 18) contained a small amount of 11SR in addition to 13SC.

13SC, but Not 8SC or 11SR, Supports ATP-Dependent Supercoiling of DNA *in Vitro*. We first tested the DNA-binding activity of each complex by a DNA-beads binding assay. 11SR, 13SC, or 8SC was purified from a mitotic extract and incubated with paramagnetic beads coupled with a 2.9-kb DNA fragment in buffer containing a range of salt concentrations. As a negative control, the same beads with no coupled DNA were used. After washing the beads with the same buffer, protein fractions bound to the beads were quantitated by immunoblotting with antibodies against condensin subunits (Fig. 3*A*). We found that 13SC efficiently bound to DNA in a buffer containing 50 mM or 100 mM KCl. 8SC also displayed a DNA-binding activity, yet weaker than 13SC: it bound to DNA at 50 mM KCl, but not at 100 mM KCl. In contrast, a much lower level of interaction was observed between 11SR and DNA even at 50 mM KCl. The presence or absence of ATP had little effect on the DNA-binding activity of each complex (data not shown). These results suggest that the SMC subunits posses a major DNA-binding activity that is strengthened by the presence of the non-SMC subunits.

We previously reported that a "13S condensin" fraction introduces positive supercoils into relaxed circular DNA in the presence of topoisomerase I and ATP (17). The current study revealed that the original fraction of 13S condensin contained a small amount of 11SR in addition to 13SC. Moreover, 8SC had never been tested for the supercoiling activity. To test which complex(es) can support the activity, relaxed circular DNA was incubated with increasing amounts of each complex in a buffer containing 50 mM KCl in the presence of topoisomerase I and ATP (Fig. 3*B*). We found that 13SC was able to introduce supercoils in a dose-dependent manner, but neither 8SC nor 11SR was able to support the reaction. Similar results were obtained for the knotting assay (ref. 18; data not shown). We then assayed the ATPase activity of each complex at two different salt concentrations (Fig. 3*C*). In an affinity-purified fraction of 13SC, we detected an ATPase activity that was stimulated ≈3 fold in the presence of double-stranded DNA at both 50 mM and 100 mM KCl. A much lower level of ATPase activity was detected in an 8SC fraction, but this activity was not stimulated by DNA even at 50 mM KCl. The ATPase activity of an affinity-purified fraction of 11SR was close to a background level. These results suggest that only 13SC can support the DNA-stimulated ATPase and ATP-dependent positive supercoiling activities *in vitro*. DNA binding by 8SC at 50 mM KCl is not sufficient either to activate its ATPase activity or to support supercoiling of DNA.

13SC, but Not 8SC or 11SR, Can Drive Chromosome Condensation in Cell-Free Extracts. We then tested which complex(es) associates with chromatin and induces chromosome condensation in the *Xenopus* egg cell-free extracts. Combinations of differential immunodepletion and add-back of affinity-purified fractions allowed us to manipulate the level of each complex in the extracts (Fig. 4*A*). A mixture of antibodies against XCAP-C, -D2, -E, and -G was used to prepare a mitotic extract depleted of all of the condensin subunits. Alternatively, 13SC and 11SR were depleted by using a mixture of antibodies against anti-XCAP-D2 and -G, producing an extract that contained 8SC only (8SC extract). Purified holocomplex or subcomplexes were then added back into these extracts. After incubating sperm chromatin with each extract for 2 h, condensin subunits bound to the chromatin were analyzed by immunoblotting (Fig. 4*B*), and chromosome morphology was examined by fluorescence microscopy (Fig. 4*C*). In a mock-depleted extract, the five subunits of condensin bound efficiently to chromatin and induced chromosome condensation. No condensation was observed in the absence of 11SR, 13SC,

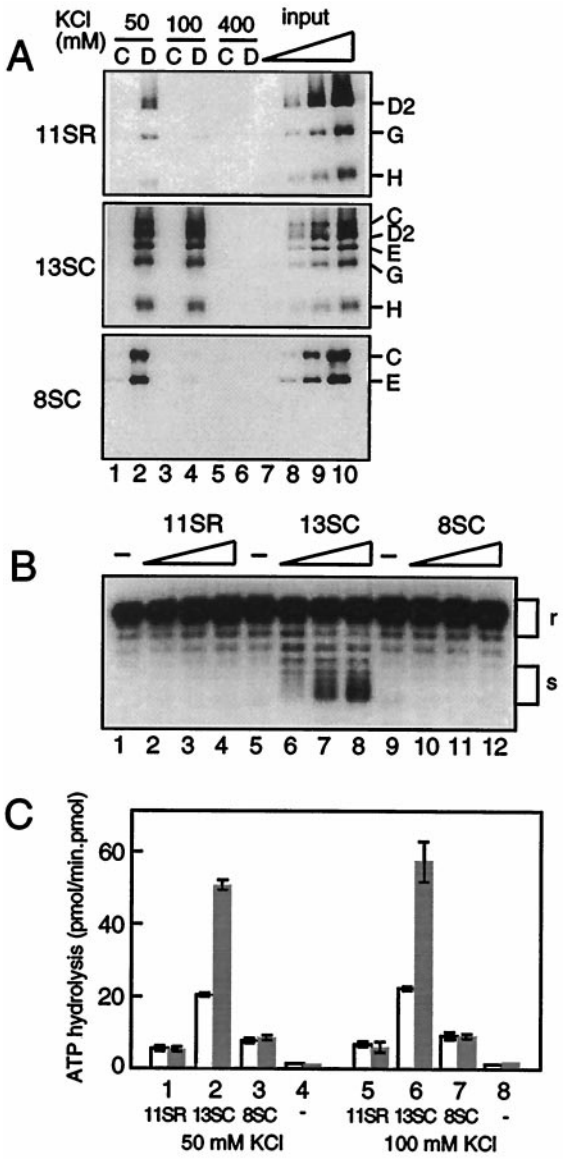

Fig. 3. Biochemical activities of condensin and its subcomplexes. (*A*) DNA-coupled (lanes 2, 4, and 6) or control (lanes 1, 3, and 5) paramagnetic beads were incubated with the same concentration of purified 11SR (*Top*), 13SC (*Middle*), or 8SC (*Bottom*) in a buffer containing 50 mM KCl (lanes 1 and 2), 100 mM KCl (lanes 3 and 4), or 400 mM KCl (lanes 5 and 6) at 22°C for 1 h. After washing the beads with the same buffer, bound proteins were analyzed by immunoblotting. As standards, 6.3% (lane 7), 12.5% (lane 8), 25% (lane 9), and 50% (lane 10) of input proteins were loaded. (*B*) Increasing amounts of 11SR, 13SC, or 8SC were incubated with a relaxed circular DNA in the presence of calf thymus topoisomerase I and 1 mM Mg-ATP in a buffer containing 50 mM KCl. DNA was purified, electrophoresed in a 0.7% agarose gel, and visualized by Southern blotting. The positions of relaxed DNA (r) and ladder of supercoiled DNA (s) are indicated. The molar ratios of complex to DNA present in the reaction mixtures were 0 (lanes 1, 5, and 9), 22.5:1 (lanes 2, 6, and 10), 45:1 (lanes 3, 7, and 11), and 90:1 (lanes 4, 8, and 12). (*C*) An affinity-purified fraction of 11SR (columns 1 and 5), 13SC (columns 2 and 6), 8SC (columns 3 and 7), or buffer alone (columns 4 and 8) was assayed for ATPase activity without DNA (open bar), or with double-stranded DNA (filled bar) at 50 mM KCl or 100 mM KCl. ATP and its hydrolysis product ADP were separated by TLC and quantitated (17). The activities are shown as pmol of hydrolyzed ATP/min·pmol of holocomplex or subcomplex.

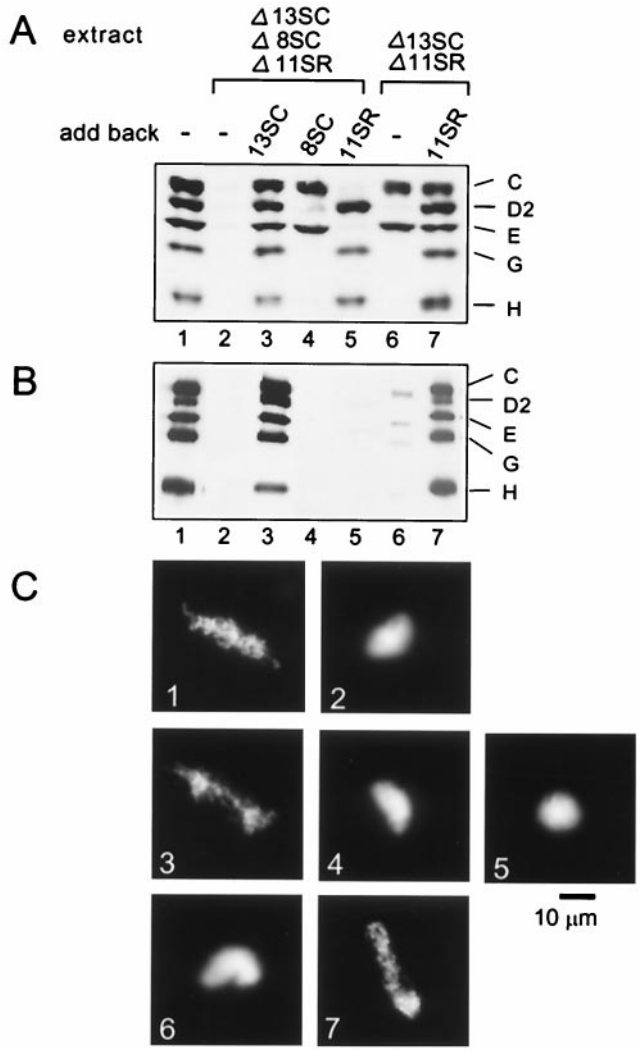

Fig. 4. Immunodepletion and rescue. (*A*) Mitotic extracts were immunodepleted with control IgG (lane 1), with a mixture of anti-XCAP-C, -D2, -E, and -G (lanes 2–5), or with a mixture of anti-XCAP-D2 and -G (lanes 6 and 7). Then a purified fraction of 13SC (lane 3), 8SC (lane 4), 11SR (lanes 5 and 7), or buffer (lanes 2 and 6) was added back into the depleted extracts. Aliquots of each extract were analyzed by immunoblotting. (*B*) After incubating sperm chromatin with each extract at 22°C for 2 h, chromatin was isolated and bound proteins were analyzed by immunoblotting. (*C*) Sperm chromatin was incubated with each extract (1–7 as in *A*) at 22°C for 2 h, fixed, and stained with 4′,6-diamidino-2-phenylindole. (Bar, 10 μm.)

and 8SC. When a purified 13SC fraction was added back into the depleted extract, about 25% of input 13SC associated with sperm chromatin and the condensation activity of the extract was largely restored. In contrast, 8SC or 11SR, when added back into the depleted extract, failed to associate with chromatin, and little sign of condensation was observed. Consistently, 8SC alone did not bind to chromatin in the 8SC extract. When 11SR was added back into this extract, however, both the SMC and non-SMC subunits bound to chromatin, leading to a substantial level of condensation. A coimmunoprecipitation experiment confirmed that the two subcomplexes interacted with each other to form a functional holocomplex in the extract (data not shown).

Cell Cycle-Specific Chromatin Assembly Around DNA-Coupled Beads. In the *Xenopus* egg cell-free extracts, mitosis-dependent chromosomal targeting of 13SC is tightly correlated with mitosis-specific phosphorylation of the non-SMC subunits (4). However, the mitotic and interphase forms of 13SC, once purified from the extracts, exhibit a similar level of binding to naked DNA (6). As

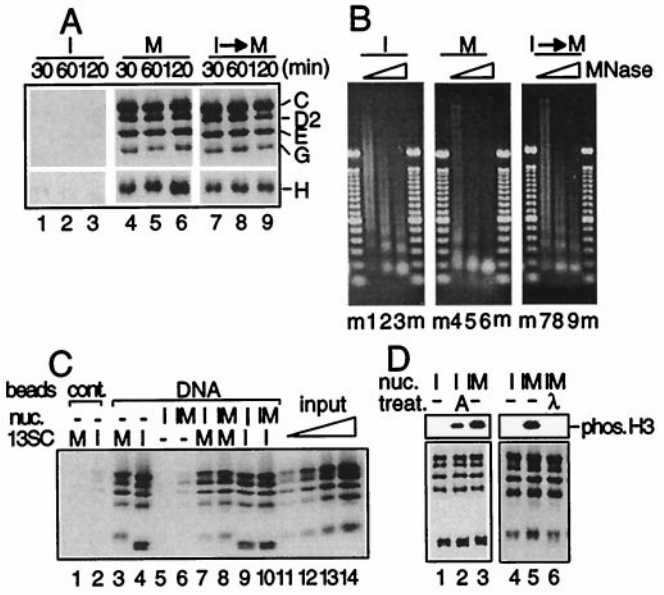

Fig. 5. Cell cycle regulation of interactions between 13SC and nucleosomes. (*A*) DNA-coupled magnetic beads were incubated with an interphase extract (lanes 1–3) or a mitotic extract (lanes 4–6) for different times as indicated. Alternatively, the DNA beads were first incubated with an interphase extract for 60 min and then with a mitotic extract for the indicated times (lanes 7–9). After washing the beads, bound proteins were analyzed by immunoblotting. (*B*) I nucleosomes, M nucleosomes, and I→M nucleosomes were assembled as shown in *A*. After washing the beads, DNA was digested with increasing amounts of micrococcal nuclease, isolated, and fractionated in a 1.25% agarose gel. m, 100-bp ladder marker. (*C*) DNA-coupled magnetic beads (lanes 4–10) or control beads (lanes 1 and 2) were incubated with buffer alone (lanes 1–4), with a condensin-depleted interphase extract for 120 min (lanes 5, 7, and 9), or with a condensin-depleted interphase extract for 60 min and then with a condensin-depleted mitotic extract for another 60 min (lanes 6, 8, and 10). The beads were washed and incubated with the mitotic form of 13SC (lanes 1, 3, 7, and 8), with the interphase form of 13SC (lanes 2, 4, 9, and 10), or with buffer alone (lanes 5 and 6) for 60 min. After washing the beads, bound 13SC was detected by immunoblotting. As standards, 6.25% (lane 11), 12.5% (lane 12), 25% (lane 13), and 50% (lane 14) of input 13SC were loaded in parallel. (*D*) Condensin-free I nucleosomes (lanes 1, 2, and 4) or I→M nucleosomes (lanes 3, 5, and 6) were assembled around DNA-coupled beads as described in *C*. The beads were washed and treated with protein kinase A (lane 2), λ phosphatase (lane 6), or control buffers (lanes 1 and 3–5). The phosphorylation states of histone H3 were analyzed by immunoblotting with a phosphospecific antibody that recognizes Ser-10 (*Upper*). Alternatively, the beads were incubated with the mitotic form of 13SC for 60 min. After washing the beads, bound proteins were analyzed by immunoblotting (*Lower*).

an initial attempt to reconstitute the mitosis-specific chromosomal binding of 13SC, we have set up a "solid-phase" chromatin assembly system (21). We first assembled chromatin around DNA-coupled paramagnetic beads in cell cycle-specific extracts and then separated it from soluble components in a magnetic field (Fig. 5*A*). We found that the five subunits of 13SC bound to the immobilized DNA in a mitotic extract, but not in an interphase extract. When the beads were first incubated with an interphase extract and then transferred to a mitotic extract, the subunits were recovered in the DNA-bound fraction. Thus, binding of 13SC to immobilized DNA faithfully reflected the cell cycle-dependent targeting to sperm chromatin (4, 22, 23). Micrococcal nuclease digestion of assembled chromatin (Fig. 5*B*) resulted in a regular array of nucleosomes in an interphase extract ("I nucleosomes"). The spacing of nucleosomes was reproducibly poor in a mitotic extract, displaying a smeared pattern of digestion ("M nucleosomes"). However, when the DNA beads were incubated in an interphase extract before exposing them to a mitotic extract, regularly spaced nucleosomes were assembled ("I→M nucleosomes"). We considered that I→M nucleosomes were more likely to represent the physiological form of mitotic nucleosomes and used them in the subsequent experiments.

Interaction of 13SC with Nucleosomes. To test cell cycle-specific interactions between purified 13SC and nucleosomes, we first assembled nucleosomes in interphase or mitotic extracts that had been depleted of condensin. The immobilized nucleosomes were isolated in a magnetic field and then mixed with 13SC purified from either interphase or mitotic extracts (Fig. 5*C*). We found that the mitotic and interphase forms of 13SC bound equally to naked DNA. Both forms of 13SC also bound, in an indistinguishable manner, to I nucleosomes, or I→M nucleosomes.

Mitosis-specific phosphorylation of histone H3 at serine 10 (Ser-10) is tightly correlated with mitotic chromosome condensation (24–26), leading to a proposal that this modification may actively recruit condensin to condensing chromatin (8, 26). To test this hypothesis directly, we manipulated the phosphorylation states of histone H3 on the immobilized nucleosomes and tested their interactions with a purified, mitotic form of 13SC. Phosphorylation at Ser-10 was mitosis-specific in this system (Fig. 5*D*, *Upper*). I nucleosomes were phosphorylated with protein kinase A (27, 28), and I→M nucleosomes were treated with λ phosphatase. We found that none of these treatments affected the efficiency of binding of purified 13SC to the nucleosomes (Fig. 5*D*, *Lower*). These results suggest that, at least in this *in vitro* system, neither phosphorylation of histone H3 nor phosphorylation of the non-SMC subunits of 13SC is sufficient to account for the mitosis-specific targeting of 13SC to chromatin.

Discussion

The 13S condensin complex (13SC) is composed of two SMC subunits and three non-SMC subunits (4, 10, 12). We reported previously that *Xenopus* egg extracts contain an 8S subcomplex (8SC) consisting of the two SMC subunits, XCAP-C and -E (4). In this study, we describe that a subpopulation of the three non-SMC subunits, XCAP-D2, -G, and -H, exists in the extracts as a separate subcomplex (11SR). Our results suggest that 13SC, but not 11SR or 8SC, is the active form that supports ATP-dependent supercoiling of DNA *in vitro* and chromosome condensation in the cell-free extracts. The physiological significance of the presence of the two subcomplexes in *Xenopus* egg extracts is unknown. They could be assembly intermediates or storage forms that are uniquely present in the early embryonic system. Alternatively, it is possible that they play more active, currently unknown, roles in chromosome assembly because at least a subcomplex similar to 8SC is present in HeLa cell nuclear extracts (T.H., unpublished observation). Consistent with the current biochemical study, recent genetic studies in yeasts underscore the importance of the non-SMC subunits of condensin *in vivo*: each of them is essential for proper condensation and segregation of mitotic chromosomes (10, 12–14). A genetic interaction between the XCAP-G and XCAP-H homologs (14) further supports the idea that the two subunits may interact directly with each other within the non-SMC subcomplex.

A DNA-coupled beads binding assay has shown that 8SC binds to DNA *in vitro*, consistent with previous work showing that SMC proteins or their truncated fragments can form protein–DNA complexes (19, 20). However, 13SC displays more stable DNA-binding than 8SC, suggesting that the non-SMC subunits enhance or stabilize the interaction between DNA and the SMC subunits. Intriguingly, only 13SC, but not 8SC, exhibits a DNA-stimulated ATPase activity under a condition where both complexes can bind to DNA. This is in striking contrast to the *Bacillus subtilis* SMC homodimer, which is active as a DNA-stimulated ATPase without any associated subunit (20). A database search

reveals that no sequences homologous to XCAP-D2, -G, or -H are encoded in the *B. subtilis* genome. Thus, the activation of SMC ATPase by non-SMC subunits is likely to be a eukaryote-specific strategy that has evolved to support more sophisticated functions of SMC protein complexes. Consistent with this idea, no ATP-dependent positive supercoiling activity has been detected in the bacterial SMC homodimer (20).

What are the specific roles of the individual non-SMC subunits in condensin regulation? Few clues are available to answer this question at this moment. It was proposed that the *Drosophila* homolog of XCAP-H, Barren, directly interacts with topoisomerase II and participates in chromosome segregation by stimulating its enzymatic activity (15). Subsequent studies, however, failed to detect evidence for physical or functional interactions between Barren and topoisomerase II (4, 13). Thus, the specific role of XCAP-H/Barren remains an open question. Sutani *et al.* (10) found that the *S. pombe* orthologs of XCAP-D2 and XCAP-G share weak similarity to AP3-β, a protein that assists the assembly of clathrin molecules. More recently, we have extended this observation and found that XCAP-D2 and XCAP-G (as well as AP3-β) share HEAT repeats, highly degenerate repeat sequences detected in a large number of proteins with diverse functions (29). Among them, a protein called cofactor D acts as a GTPase activating protein that stimulates β-tubulin to hydrolyze GTP (30). Likewise, the HEAT domain of elongation factor-3 (EF-3) binds to the ribosome and stimulates its own ribosome-dependent ATPase activity (31). Taking these observations together with our biochemical data, it is tempting to speculate that the HEAT domains of XCAP-G and XCAP-D2 are directly involved in the activation of SMC ATPases. Alternatively, they could play an architectual role to help proper assembly of the condensin complex (10) or to convert the SMC heterodimer into a functional conformation.

Our results suggest that both the SMC and non-SMC subunits are required to confer mitosis-specific chromosomal association of condensin in the cell-free extracts. We have also shown that neither phosphorylation of the non-SMC subunits nor that of histone H3 at Ser-10 is sufficient to account for this level of regulation. Consistently, a recent study in *Xenopus* egg extracts has shown that 13SC can interact with native and tailless nucleosomes with similar efficiency (32). It is therefore most likely that a soluble factor(s) present in the extracts helps establish the mitosis-specific chromosomal targeting of 13SC. Mitotic extracts may contain a loading factor that recognizes the phosphorylated form of the non-SMC subunits and actively recruits 13SC to chromosomes. Alternatively, there may be an inhibitor that prevents 13SC from associating with chromatin in interphase extracts. Very recently, an A kinase-anchoring protein, AKAP95, has been identified as a potential candidate for a chromosomal receptor that assists loading of condensin in HeLa cells (33). The role of AKAP in the *Xenopus* egg extracts remains to be determined. The solid-phase chromatin assembly system described in this paper will be powerful for identifying the putative loading or unloading factors for condensin and studying their cell cycle regulation *in vitro*.

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