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**Molecular and Genetic Analysis of Condensin Function in Vertebrate Cells**

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We engineered mutants into residues of SMC2 to dissect the role of ATPase function in the condensin complex. These residues are predicted to be involved in ATP binding or hydrolysis and in the Q-loop, which is thought to act as a mediator of conformational changes induced by substrate binding. All the engineered ATPase mutations resulted in lethality when introduced into SMC2 null cells. We found that ATP binding, but not hydrolysis, is essential to allow stable condensin association with chromosomes. How SMC proteins bind and interact with DNA is still a major question. Cohesin may form a ring structure that topologically encircles DNA. We examined whether condensin behaves in an analogous way to its cohesin counterpart, and we have generated a cleavable form of biologically active condensin with PreScission protease sites engineered into the SMC2 protein. This has allowed us to demonstrate that topological integrity of the SMC2-SMC4 heterodimer is not necessary for the stability of the condensin complex in vitro or for its stable association with mitotic chromosomes. Thus, despite their similar molecular organization, condensin and cohesin exhibit fundamental differences in their structure and function.

**INTRODUCTION**

The assembly of DNA into condensed chromosomes during mitosis is essential for the faithful segregation of the genome into daughter nuclei. Several studies have shown that the condensin complex has a crucial role in the formation of structurally stable mitotic chromosomes and their segregation in vivo (Strunnikov et al., 1995; Hagsstrom et al., 2002; Hudson et al., 2003; Hirano, 2005). Although vertebrate chromosomes lacking condensin manage to compact their chromatin almost normally, they lose their organized architecture during anaphase as Repo-Man targets protein phosphatase 1 to the separating chromosomes (Vagnarelli et al., 2006). Thus, one role of the condensin complex is to direct and/or regulate the association of other nonhistone proteins with mitotic chromosomes. Although the requirement for condensin function in chromosome architecture is well established, its mechanism of action remains an open question.

The two forms of condensin, condensin I and II, are pentameric complexes composed of the SMC2 and SMC4 ATPases plus three auxiliary subunits (CapG/G2, CapD2/D3, and CapH/H2) (Ono et al., 2003; Hirota et al., 2004). The two SMC subunits are known to be responsible for ATPase activity, which is essential for condensin function (Stray and Lindsley, 2003). However it is not known how the ATPase activity contributes to chromosome condensation.

Condensin subunit ScII/SMC2, first isolated as a component of the chromosome scaffold fraction (Saitoh et al., 1994), belongs to the structural maintenance of chromosome (SMC) family, a large family of chromosomal ATPases involved in chromosome condensation, sister chromatid cohesion, and DNA repair (Cobbe and Heck, 2000; Hirano, 2006). These proteins share aspects of common architecture with ATP-binding cassette (ABC) membrane transporters (Saitoh et al., 1995; Hopfner et al., 2001). Both contain Walker A and B consensus sequences as well as the highly conserved ‘LS-GGQ’ signature sequence or C-motif. In DNA mismatch repair, the MutS ABC ATPase uses ATP binding to recognize and bind misrepaired DNA (Junop et al., 2001). In double-stranded break repair, Rad50 uses ATP to bind and bridge DNA double-strand breaks (Chen et al., 2001; Lobachev et al., 2002), and ABC transporters use the energy from ATP hydrolysis to transport substances across membranes (Hyde et al., 1990).

A common theme has emerged among ABC-like ATPases, despite the wide range of functions carried out by this diverse protein family. ATP binding through the signature and Q-loop motifs causes conformational changes necessary to accomplish functions as diverse as DNA repair and transmembrane transport (Hopfner and Tainer, 2003). Although several studies indicate that ATPase activity of condensin is needed for its enzymatic properties, the precise role of ATP binding and hydrolysis is not yet known. In vitro studies in Xenopus egg extracts show that positive knotting and super-
coiling of plasmid DNA by high concentrations of condensin are ATP dependent (Kimura and Hirano, 1997; Kimura et al., 1998), and studies using nano-manipulation of a single DNA molecule show that condensin I can compact DNA in an ATP hydrolysis-dependent manner (Bazett-Jones et al., 2002).

Here, we report the in vivo analysis of condensin ATPase function by using a systematic mutagenesis of all known SMC2 ATPase domains, including the Q-loop, signature motif, and Walker A and B motifs. We have also generated the first biologically active cleavable form of SMC2, allowing us to break the putative condensin complex ring in vitro and determine whether condensin might function in an analogous way to cohesin by topologically embracing DNA (Gruber et al., 2003; Ivanov and Nasmyth, 2005). These studies were performed in DT40 cells with a conditional knockout of the SMC2 gene, so that all phenotypes observed reflect the activity of a homogeneous mutant complex, with no background of the wild-type protein.

Our results reveal several fundamental and intriguing differences between the condensin and cohesin complexes. We demonstrate that SMC2 ATP binding, but not hydrolysis, is required for condensin to stably associate with mitotic chromosomes. However, ATP binding is not required for formation of the condensin complex. We also show that disruption of the putative condensin ring does not affect the integrity of the complex or its ability to associate with mitotic chromosomes.

MATERIALS AND METHODS

Cell Culture and Transfections

The chicken DT40 cell culture and repression experiments in the SMC2 conditional knockdown cell line were performed as described previously (Hudson et al., 2003; Vagnarelli et al., 2006). For transient transfections (Figure 3A), 1.0 x 10^6 DT40 cells were rinsed with phosphate-buffered saline (PBS) and then suspended into 0.1 ml of nucleotector solution V per cuvette. DNA (2–10 μg) was added to each cuvette and electroporated (Program B-30, Nucleofector II, Amaxa, Cologne, Germany).

Construction of SMC2 ATPase Mutants and PreScission SMC2

Wild-type SMC2 cDNA, SMC2 PreScission, and SMC2 PreScission mutants were cloned downstream of the 3×His base pairs SMC2 promoter fragment. Point mutations and general ATPase mutations were introduced into the SMC2 cDNA by using a QuiChang XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by sequencing. The 45-amino acid streptavidin binding peptide (Keefe et al., 2001) for experiments in Figures 2 and 3 was linked to the C terminus of SMC2 by using a unique SalI site engineered over the stop codon. The TrAP tag for the PreScission SMC2 construct was also inserted at the SalI site. To generate SMC2 PreScission, oligonucleotides containing the eight-amino acid (Leu Glu Val Leu Phe Gln Gly Pro) PreScission protease recognition sites were cloned into SMC2 at amino acid position 386 and 949 by using a unique AvrII site engineered into the SMC2 cDNA with QuiChang XL under the control of the SMC2 3.8-kb promoter fragment. Oligonucleotide sequences used for mutagenesis, insertion of PreScission sites, streptavidin binding peptide (SBP), TrAP tags, and cloning of SMC2 promoter are described in Supplemental Data.

Condensin Affinity Purification

SBP-tagged or SBP/His/S-tagged condensin was isolated from DT40 cells by using streptavidin beads (Chemical Pierce, Rockford, IL) for the SBP tag and S-protein agarose beads (Novagen, Darmstadt, Germany) for the S tag. In general, 10^6 colcemid-blocked cells were frozen as cell pellets and stored at −80°C. Lysis was performed on thawed cell pellets by using 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% NP-40, 1 mM CaCl2, 30 μg/ml RNAse A, 40 μg/ml micrococcal nuclease, 1 μg/ml aprotinin, 1 μg/ml leupeptin, antipain, and pepstatin A, and 1 mM phenylmethylsulfonyl fluoride for 45 min on ice followed by the addition of EDTA and deoxycholate to 1 mM and 0.1%, respectively. Lysates were centrifuged at 4°C for 10 min at 14,000 rpm. Streptavidin agarose beads (1 ml) were mixed with cleared lysates (supernatant) for 2 h, rotatating at 4°C in a final volume of 10 ml. Beads were washed in wash buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, or 250 mM NaCl for PreScission assays, 0.5% NP-40) three times and eluted in elution buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl for Figure 2, C and D or 250 NaCl mM for Figure 5), 0.5% NP-40, 0.1% deoxycholate, and 4 mM biotin). For Figure 2C, the sample was eluted from streptavidin beads by boiling in SDS sample buffer to enable full Affinity purification (Figure 5, 1 and J), the beads were then added to 700 μl of 0.5-protein agarose beads (Sigma-Aldrich) in 1 ml of S-protein agarose beads. Subsequent binding and digestion conditions were as for streptavidin beads with sample eluted from S-beads by boiling in SDS sample buffer.

Preparation of Mitotic Chromosome and Scaffolds

Somatic cell nuclei (SCN) in vitro by using the three-dimensional decondensation method (Lewis and Laemmli, 1982). The insoluble scaffold fraction was pelleted at 6800 x g and solubilized in SDS sample buffer.

PreScission Protease Digestion

Isolated mitotic chromosomes were treated with PreScission protease for 16 h at 4°C with 80 μl of enzyme (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), and scaffolds were isolated as described. For pull-down isolation assays for condensin bound to streptavidin beads a further two times in PreScission buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.01% Triton, and 1 mM EDTA) and suspended in 1-ml final volume of the same buffer. Digestion was performed for 16 h on a rotating platform at 4°C with 80 μl of enzyme.

Immunoblotting Analysis

Protein samples for total lysate, affinity-purified condensin, and from isolated chromosomes were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose membrane (GE Healthcare). After blotting, the membranes were stained with Ponceau S (Sigma-Aldrich, St. Louis, MO); Membranes were washed with 5% skim milk in PBS and processed for enhanced chemiluminescence by standard methods. Antibodies used were as follows: rabbit anti-KIF4A at 1:1,000, mouse anti-tubulin B512 Sigma-Aldrich) at 1:1,000, anti-SMC4 (Acris Antibodies, Hiddenhausen, Germany) at 1:500, anti-ScI/H (Sigma-Aldrich) at 1:1,000, and monocular anti-SBP (1:300). The Gallow gallus CAP-D2 antibody was raised to a 28-kDa peptide fragment in rabbit, corresponding to amino acids 1077–1324, and it was used at 1:1,000.

Quantification of Antigen Recovery in the Scaffolds and Supernatant Fractions

Scaffold and supernatant samples were prepared as described above and boiled in SDS-sample buffer. A range of volumes of these samples was then subjected to SDS-PAGE in 7.5% polyacrylamide gels, and immunoblots were used to determine the amount of each antigen present. The intensities corresponding to each antigen were quantified using ImageJ (NIH Image; http://rsb.info.nih.gov/ij/). To calculate the protein recovery in the scaffold and supernatant fractions, standard curves were made from at least four points corresponding to different loading volumes. Experimental values were then extrapolated from the linear portion of these curves. The percentage of each protein recovered in the scaffold fraction was calculated as follows: [scaffold/(scaffold + supernatant)] x 100.

Indirect Immunofluorescence Microscopy

Chromosome spreads were prepared from cells either dropped onto slides (Figure 2B and Supplemental Figure S3) or grown on coverslips (Figures 3A and 4A) and processed as follows. Cultured cells were blocked in mitosis with colcemid (100 ng/ml) for 2 h, hypotonically swollen in 75 mM KCl for 5 min, and fixed with cold methanol-acetic acid (−20°C) (3:1). Primary antibodies (anti-KIF4A at 1:500, SMC2 M at 1:200, CAP-D2 at 1:200, CAP-H-1 at 1:200, CAP-H-2 at 1:200, CAP-D2 at 1:200, and SBP at 1:50) in TEEN buffer (1 mM triethanolamine-HCl, pH 8.5, 0.2 mM Na-EDTA, and 25 mM NaCl) with 0.1% Triton and 1% bovine serum albumin (BSA) were incubated for 30 min. Cells were washed three times with TB buffer (10 mM Tris-HCl, pH 7.7, 150 mM NaCl, and 0.1% BSA), and fluorescence-labeled secondary antibodies were applied (Invitrogen, Carlsbad, CA; for Figure 4 at 1:50; Jackson Immunoresearch Laboratories, West Grove, PA; Figures 2B and 3A and Supplemental Figure 2 at 1:200) and counterstained with 4,6-diamidino-2-phenylindole (Calbiochem, Darmstadt, Germany). Three-dimensional data sets were collected with a DeltaVision system (Applied Precision, Issaquah, WA) based on an IX-70 inverted microscope (Olympus, Tokyo, Japan) with a Sedat filter set (Chroma Tech-
RESULTS

ATP Binding and Hydrolysis Are Required for SMC2 Function

We introduced mutations into four conserved domains of SMC2 implicated in ATPase activity (Walker A, Q-loop, Signature motif, and Walker B) (Figure 1, A and B). All mutants were expressed in SMC2ON/OFF conditional knockout cells under control of a promoter fragment consisting of the 3.8-kb genomic fragment directly upstream of the start codon of SMC2. This allowed the phenotype of the mutant proteins to be analyzed in the absence of endogenous SMC2. Wild-type SMC2 expression driven by this promoter fragment complemented SMC2OFF cells (Supplemental Figure S1, A and B).

Our mutagenesis strategy was based on the premise that the mutations would be expected to have the same effect in SMC2 as they do in bacterial SMC proteins (Hirano et al., 2001; Hirano and Hirano, 2006), cohesin SMCs (Arumugam et al., 2003; Weitzer et al., 2003; Arumugam et al., 2006) ABC transporters and RAD 50 (Hopfner et al., 2000; also see Figure 1. ATP binding and hydrolysis are required for SMC2 function. (A) Sequence alignment using MUSCLE (Edgar, 2004) of conserved SMC ATPase domains from G. gallus SMC2 (NP_995651.1), B. subtilis SMC (NP_389476.1), Rhodobacter ferriredoxins SMC (ABD69231.1), Saccharomyces cerevisiae SMC3 (NP_012461.1), S. cerevisiae SMC2 (NP_116687.1), Ciona savignyi SMC (ENSCAVP00000005455), and Homo sapiens SMC2 (NP_006435.2). Site-directed mutagenesis positions are highlighted in red. (B) Mutations engineered into SMC2 and their expected effects on ATPase activity.

nology, Brattleboro, VT) driven by the SoftWoRx software under standard conditions. All the image files were captured as raw (r3d) files and deconvolved (d3d) files subsequently prepared by the SoftWoRx software (Applied Precision) deconvolution algorithm. Three-dimensional data sets were converted to Quick Projections in SoftWoRx and then imported into Adobe Photoshop (Adobe Systems, Mountain View, CA) for final presentation. Levels were adjusted across each entire image to lower nonspecific background haze using the standard Photoshop adjustment of levels.
Figure 2. SMC2 ATPase activity is not essential for the formation of the condensin I holocomplex. (A) SBP-tagged SMC2 is able to rescue SMC2OFF cells. SMC2 conditional knockout cells (5 × 10⁶ cells, SMC2OFF, SMC2OFFSBP and SMC2OFFSBPmutant and SMC2OFFSBPWT were resolved by 10% SDS-PAGE and immunoblotted with anti-SBP. Proteins from equivalent amounts of cells were added (Figure 2A). Chromosomes containing only SMC2SBP protein after doxycycline was added (Figure 2A). Chromosomes containing only SMC2SBP had normal morphology and displayed the characteristic SMC2 axial staining (Figure 2B). Thus, SMC2SBP seems to be a fully functional protein.

To assess whether ATPase mutations of SMC2 affect formation of the condensin complex, pull-downs were performed from stable cell lines expressing the wild type (WT), K38I, D1113A, E1114Q and Q147L ATPase mutants tagged with SBP. Proteins from equivalent amounts of cells were silver stained (Figure 2C) or immunoblotted for the condensin I components CAP-D2 and CAP-H (Figure 2D). Silver stain analysis revealed that SMC2-SMC4 heterodimer formation was not affected by any of the mutations, and immunoblotting with CAP-D2 and CAP-H antibodies indicated that the non-SMC subunits of condensin I assembled normally with all of the various mutants. This agrees with in vitro data using baculovirus expressed condensin I (Omm et al., 2007). Thus, a functional condensin ATPase is not a prerequisite for assembly of the complex in vivo.

SMC2 ATP Binding but Not Hydrolysis Is Essential for Loading Condensin onto Mitotic Chromosomes

Given that the condensin complex formed, one explanation for the failure of SMC2 ATPase mutations to rescue condensin function might be that ATP binding and/or hydrolysis is required for the complex to assemble onto mitotic chromosomes. To determine whether the various mutant condensin complexes could still load onto mitotic chromosomes, SMC2OFFSBP cells were transiently transfected with vectors expressing wild-type or mutant SMC2 10 h after addition of doxycycline to turn off the conditional wild-type allele. Transfected cells were allowed to express the various SMC2 mutants for 16 h, hypotonically swollen, and then fixed with methylamine/acidic acid (3:1) and stained with anti-SBP.

Mutations affecting ATP binding (D1113A, K38I) as well as the Q-loop mutant (Q147L) prevented any detectable loading of condensin onto mitotic chromosomes. Instead, a diffuse cytoplasmic staining was observed. In contrast, con-

Summary in Figure 1B). A Walker A box mutation, K38I, and the D1113A mutation in the Walker B motif are targeted to prevent ATP binding; whereas an E1114Q “transition state” mutation in the Walker B box would slow the rate of ATP hydrolysis that is thought to be required for head disengagement in SMC complexes (Hirano, 2006). The S1086R mutation in the C-terminal signature motif is predicted to allow ATP binding but prevent ATP hydrolysis. These four residues are completely conserved among SMCs and ABC transporters and have been extensively characterized (Hirano et al., 2001; Arumugam et al., 2003, 2006; Weitzer et al., 2003; Hirano and Hirano, 2006), but little is known of their role in the condensin complex in vivo. Mutation of the conserved Q-loop (Q147L) is predicted to block binding of the attacking nucleophile (water) and Mg²⁺ ions involved in ATP hydrolysis (Hopfner et al., 2000). It has been suggested that contacts between the Q-loop glutamine, and the active site magnesium and nucleophile make it a good candidate for the transmission of allosteric changes caused by substrate binding (Hopfner and Tainer, 2003). However, no SMC Q-loop mutant has been characterized in vivo in detail to date.

SMC2ON/Off cell lines stably expressing the above-mentioned mutants were isolated for further analysis. Clones were selected on the basis that they expressed levels of SMC2 cDNA similar to those in wild-type DT40 cells as determined using semiquantitative polymerase chain reaction (PCR) (data not shown). SMC2 mutants were not tagged to exclude the low possibility that the tag might produce an additive affect in combination with the mutation (note that tagged wild-type SMC2 fully rescues SMC2 function). In multiple transfections of constructs expressing the various SMC2 ATPase mutations, no clones were isolated that were able to rescue life in SMC2OFF cells. Instead, all resulting cell lines gave mitotic phenotypes similar to those of SMC2OFF cells when scored for anaphase and telophase chromosome bridges, mitotic index, multipolar spindles, and cytokinesis bridges (Supplemental Figure S1, C–F). Thus, all residues mutagenized are essential for SMC2 function.

SMC2 ATPase Activity Is Not Required for Formation of the Condensin I Holocomplex

To analyze the effect of the above mutations on condensin complex assembly, SMC2 wild-type and mutant constructs were tagged with the 45-amino acid SBP, and stable cell lines were generated containing mutant or wild-type SMC2SBP in the conditional SMC2ON/OFF background. SBP-tagged wild-type SMC2 expressed from the endogenous SMC2 promoter complemented SMC2 null cells and immunoblotting analysis revealed only SMC2SBP protein after doxycycline was added (Figure 2A). Chromosomes containing only SMC2SBP had normal morphology and displayed the characteristic SMC2 axial staining (Figure 2B). Thus, SMC2SBP seems to be a fully functional protein.

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densin complexes containing SMC2 mutations affecting ATP hydrolysis (E1114Q and S1086R) did load onto chromosomes (Figure 3A). The transition state E1114Q mutant seemed to load most strongly onto the chromosomes, whereas some diffuse staining in the cytoplasm of mitotic cells was still seen in cells expressing the S1086R mutant. Importantly, although condensin complexes containing these two mutant forms of SMC2 bound to chromosomes, SMC2 was dispersed throughout the mitotic chromatin rather than showing the normal axial staining, and chromosome structure seemed aberrant, reminiscent of SMC2 OFF chromosomes after similar treatments.

To confirm that these results were not due to masking of epitopes, we isolated mitotic chromosomes from stable cell lines expressing the SBP-tagged mutants. Blotting using anti-SBP antibody confirmed the results of the immunofluorescence analysis (Figure 3B). SMC2-E1114Q bound to chromosomes at levels comparable with wild type. SMC2-S1086R was also detected on chromosomes, albeit at lower levels. These results suggest that ATP binding might either cause a
conformational change or allow association with another factor necessary to enable condensin to load onto mitotic chromosomes.

**Topological Integrity of the SMC2-SMC4 Heterodimer Is Not Required for the Stability of Condensin I and Condensin II Complexes**

Studies of cohesin using a tobacco etch virus-cleavable subunit have led to a model in which cohesin functions as a ring that embraces both sister chromatids (Gruber et al., 2003; Ivanov and Nasmyth, 2005). To determine whether condensin also functions as a ring, albeit within rather than between sister chromatids, we constructed a form of SMC2 that could be cleaved by PreScission protease at two balanced sites in the coiled-coil region.

The primary sequence of SMC2 revealed two short stretches of low probability of coiled-coil formation within the arms of SMC2 (Figure 4B). These were chosen as sites for insertion of the eight-amino acid PreScission recognition sequence. Subsequent cleavage would generate three SMC2 fragments (Figure 4C and D). A triple affinity tag containing S/SBP and HIS tags was inserted at the C-terminal end of SMC2 to allow affinity purification of the condensin complex containing cleavable SMC2. Remarkably, this cleavable SMC2 construct was able to sustain life and complement SMC2OFF cells. Mitotic chromosome spreads from SMC2OFF/SMC2PRESCISSION cells showed normal chromosome morphology and SMC2 staining (Figure 4A). Thus, TrAP-tagged SMC2 with PreScission protease sites inserted in its arms is fully functional for condensin complex assembly and function in vivo.

To determine the effect of cleavage of the SMC2 arms on the integrity of the condensin complex, complexes containing cleavable condensin were isolated via the TrAP tags and bound to streptavidin beads (Figure 5, B–H). Added PreScission protease efficiently cleaved SMC2 into its middle, C, and N-terminal fragments (Figure 5, B–D), which corresponded to predicted sizes (Figure 4D). After stringent washing, all three fragments remained attached to the beads. The N and C terminal fragments were most resistant to washing; some of the middle fragment was released into washes and supernatant (Figure 5B). This suggests that head-to-head interaction of the SMC proteins may be stronger than interactions involving the dimerization region. Remarkably CAP-H and CAP-D2 binding was virtually identical between control and PreScission digested fractions and only a small fraction of SMC4 was lost (Figure 5, E–G, respectively).

An alternative approach was used to more rigorously test the stability of the complex containing cleaved SMC2 (Figure 5I). Cleavable condensin bound to streptavidin beads was treated with PreScission protease, washed with a buffer containing the ionic detergent deoxycholate, and then subsequently eluted using biotin. The eluted fraction was then rebound to S protein agarose beads, washed as before, and the fraction remaining on the beads was characterized. Silver stain analysis revealed that all three SMC2 fragments remained associated after two purification steps. Mass spec-
Figure 5. Integrity of the SMC2-SMC4 heterodimer is not required for the stability of condensin I and condensin II complexes. (A) Depiction of the condensin complex as a ring structure where the non-SMC subunits close the ring. Addition of PreScission protease would break this structure. A triple affinity tag (S/SBP/His) at the C terminus of SMC2 allows affinity purification. (B-H) Analysis of pull-downs from condensin isolated from SMC2OFF/SMC2PRESCISSON cells. Lysates were bound to streptavidin beads by using the SBP-tag and then PreScission was added. After the supernatant (Sup) was retained, the beads were washed three times (W1, W2, W3 [not shown]), and the sample remaining on the streptavidin beads (Str) was eluted by boiling in sample buffer. Immunoblotting was then performed using an antibody to the middle region of SMC2 (B), the C-terminal using anti-SBP (C), the N-terminal (D), CAP-H (E), CAP-D2 (F), SMC4 (G), and the corresponding samples were silver stained (H). A more stringent approach was next applied using tandem affinity purification technology. (I) Condensin was bound to streptavidin beads, cleaved by addition of PreScission protease, and eluted with biotin after three washes. The C-terminal fragment of SMC2 was then rebound to S-protein agarose beads and eluted by boiling in sample buffer. The biotin elution from Str, Sup, and S protein agarose (S) elution were silver stained. (J) The ratio of peptide number for condensin subunits from the streptavidin bead elutions (Str) and S protein agarose elutions (S) plus and minus PreScission protease showed no significant loss of either condensin I or II subunits. Data were taken from four experiments. Even after two purification steps (S elution), there is no significant loss in condensin subunits (also see Supplemental Figure S2).

only slight reductions in the levels of associated SMC2, SMC4, and CAP-H (Figure 6, B–D), suggesting the complex is remarkably stable once assembled into mitotic chromatin. Immunofluorescence of purified chromosomes treated with PreScission protease also showed no obvious differences in staining using the scaffold marker KIF4A and the SBP antibody (Figure 6G). Chromosomes purified and treated with PreScission protease retained similar architecture, and they seemed to have axial staining characteristics similar to mitotic chromosomes. This appearance is distinct from chromosomes lacking SMC2, in which scaffold markers become diffuse and chromosome architecture becomes abnormal (Hudson et al., 2003) (Supplemental Figure S3).

Previous analysis of scaffold fractions from chromosomes lacking SMC2 showed that the presence of condensin is essential for scaffold integrity (Hudson et al., 2003). Known scaffold components were absent from chromosome scaffolds isolated from SMC2OFF cells as revealed by immunoblotting and the total recovery of Coomassie-stained proteins in the scaffold fractions were also significantly reduced. Although condensin components seemed normal in the scaffold fraction after condensin was cleaved by PreScission protease, there was a substantial (>50%) increase in the solubilization of the major chromosome scaffold component DNA Topo IIα (Figure 6, E and H, and Supplemental Figure S4). The change in topoisomerase IIα solubility was selective, as no significant change was seen in the level of scaffold-associated kinesin KIF4A (Figure 6, F and H, and Supplemental Figure S4). The overall protein content in the supernatant fraction of scaffolds for chromosomes treated with PreScission was also significantly increased (Figure 6A, lane 6). Thus, although the scaffold fraction is retained when condensin is cleared, the overall architecture is compromised leading to destabilization of individual scaffold components.

DISCUSSION

Cohesin and condensin are the two most widely studied nonhistone protein complexes with important roles in mitotic chromosome structure and behavior. The present study serves to highlight that even though these two complexes are superficially similar (e.g., both are built around SMC protein heterodimers), the role played by ATP in the function of the two complexes is apparently distinct.

Mechanistic Differences Between Cohesin and Condensin ATPase Function

Our study shows ATP binding and hydrolysis by SMC2 are not required for the assembly of the condensin holocomplex. A separate study using baculovirus purified condensin I also found that ATPase mutations in either SMC2 or SMC4 failed to affect the formation of the holocomplex in vitro (Omm et al., 2007). In contrast, ATP binding in the cohesin subunit SMC1 but not SMC3 is essential for interactions with Scc1 and therefore for assembly of the cohesin complex (Arumugam et al., 2003; Weitzer et al., 2003).

We also report the first functional analysis of the conserved Q-loop mutant Q147L found in all SMC proteins. This motif has been proposed to bind water and magnesium and to undergo a conformational change upon substrate binding (Hopfner et al., 2000; Hopfner and Tainer, 2003). It has been suggested that this acts as a “lever arm” to transduce ATP binding and hydrolysis into physical action by the SMC protein (Hopfner and Tainer, 2003). We found that SMC2 Q-loop mutant Q147L has no effect on the formation of the condensin holocomplex, but abolished its association with
chromosomes. It remains to be determined whether this Q-loop mutant has impaired ATPase function or acts downstream of ATP to disrupt a conformational change required for condensin loading.

ATP-binding, but not hydrolysis is required for the association of condensin with mitotic chromosomes in vivo. Despite the fact that SMC2 mutants D1113A and K38I, which are predicted to block ATP binding, are able to participate in formation of the condensin complex, complexes containing these mutations failed to associate with chromosomes. In contrast, the SMC2-E1114Q mutant, which is predicted to slow the rate of ATP hydrolysis, bound to chromosomes at levels comparable with wild type. The SMC2-S1086R mutant bound chromosomes less well, possibly reflecting its ability to bind, but not to hydrolyze, ATP. These results are consistent with those of an in vitro study of Bacillus subtilis SMC homodimers, in which transition state mutants (analogous to SMC2-E1114Q) allowed detectable DNA binding (Hirano and Hirano, 2004). In contrast, cohesin complexes with the analogous ATP hydrolysis mutation in either SMC1 or SMC3 fail to load onto chromatin (Arumugam et al., 2003).

Our in vivo studies of condensin function are consistent with recent in vitro studies from the Hirano laboratory, which showed that purified SMC2 undergoes a conformational shift in the presence of ATP, leading to the suggestion that ATP binding might open the hinge region (Onn et al., 2007). Recent studies of cohesin have shown that loading of the complex onto chromatin is caused by transient opening of the hinge domain, and it was hypothesized that this conformational change could be the result of either ATP binding or hydrolysis (Gruber et al., 2006). Thus, it seems that ATPase activity within the head domain could relay the conformational changes required to open the hinge region for SMC proteins (Figure 7).

Regardless of the mechanism, cells expressing the mutated ATPase domains in SMC2 are unable to form functional condensin complex. Although the results reported here represent a first attempt using a genetic approach to understanding the role of condensin ATPase function in vivo, further work is required for a definitive characterization of the ATPase cycle in purified condensin.

**Cleavage of SMC2 Does Not Alter Condensin Association or Binding to Chromosomes in Vitro**

One key to understanding the function of condensin is to ascertain whether the complex forms a closed ring structure and traps DNA in a manner analogous to that proposed for cohesin (Ivanov and Nasmyth, 2005). Electron microscopy studies reveal that the cohesin arms seem to form an open loop, and they are thus topologically in a position to encircle DNA, whereas condensin predominately forms a “lollipop” like structure with the arms tightly apposed to one another (Anderson et al., 2002). Our observations that the condensin complex remains largely intact despite the cleavage of SMC2 are consistent with the notion that condensin arms are apposed in a lollipop structure. Our results thus support the notion that condensin acts via a mechanism distinct from cohesin.

By analogy to experiments with the cohesin subunit SMC3 (Gruber et al., 2003), cleavage sites were chosen that would break SMC2 in regions of lowered propensity for coiled-coil formation and therefore would not interfere with the structure of the complex. However, we cannot exclude the possibility that interactions between SMC2 helices (or with
SMC4) in the coiled-coil might be retained even after protease cleavage. After in vitro cleavage of SMC2 by PreScission protease, a significant portion of the middle hinge/dimerization region could be substantially solubilized (Figure 5B), consistent with cleavage rather than simply “nicking” the SMC2 coiled-coil, whereas the C and N domains remain tightly associated (Figure 5, C and D). In the previous study of cohesin, when cleavable SMC3 was expressed and cleaved in vitro on beads, approximately half of the dimerization domain was released, suggesting comparable cleavage of SMC proteins between the two systems (Gruber et al., 2003). However, given the predicted lollipop conformation for the condensin holocomplex, we cannot say whether cleavages within the SMC2 coiled-coil open the complex entirely when the complex is bound to chromosomes.

Cleavage of SMC3 releases the cohesin complex from chromatin and can initiate the onset of sister chromatid separation, even though it does not alter the interactions between SMC3, SMC1, or Sccl (Gruber et al., 2003). When SMC2 in purified condensin is cleaved by PreScission protease, the complex seems to remain intact without any significant loss of either the condensin I or II non-SMC subunits. This was true even under stringent tandem purification conditions with multiple washes in a buffer that included the ionic detergent deoxycholate. Furthermore, when isolated chromosomes were treated with PreScission protease, SMC2 remained concentrated along the chromatid axes despite being quantitatively cleaved. Therefore, condensin complex stability and association with mitotic chromosomes is not dependent upon the integrity of the SMC2 heterodimer. In contrast, the chromosome association of DNA topoisomerase IIα was specifically altered after SMC2 cleavage. This demonstrates that the PreScission cleavage of SMC2 did indeed alter condensin structure or function, and it suggests a close association of Topo IIα with the condensin complex in chromosomes.

Cleavage of the SMC2 coils might be expected to release the complex from chromosome if condensin were to bind DNA by an “embrace” model as proposed for cohesin. However, the failure to release the complex from chromosomes after SMC2 scission suggests that chromosome association by condensin may not solely require topological closure of the complex. Thus, the SMC arms of condensin might transmit conformational changes that enable loading or unloading of the complex.

To date the only direct visualization of condensin associated with DNA was provided by atomic force microscopy of the purified fission yeast complex (Yoshimura et al., 2003). The work showed condensin as sitting on DNA with its hinge but not topologically embracing DNA, and in some instances with the heads bending down to the DNA. It is possible, however, these images represent condensin trapped in a preloading state because of the limited biochemical activity of the preparation or absence of loading factors (Uhlmann and Hopfner, 2006).

The way in which condensin interacts with DNA therefore remains an open question. Our in vivo data have demonstrated the importance of the ATPase cycle of condensin in regulating this process. Together, our work and the work performed by others have served to highlight a SMC paradox in which remarkably similar proteins that form highly analogous complexes seem to function by distinct mechanisms.

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