Identification and Characterization of SA/Scc3p Subunits in the Xenopus and Human Cohesin Complexes

Ana Losada, Tomoki Yokochi, Ryuji Kobayashi, and Tatsuya Hirano

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Abstract. A multisubunit protein complex, termed cohesin, plays an essential role in sister chromatid cohesion in yeast and in Xenopus laevis cell-free extracts. We report here that two distinct cohesin complexes exist in Xenopus egg extracts. A 14S complex (x-cohesinSA1) contains XSMC1, XSMC3, XRAD21, and a newly identified subunit, XSA1. In a second 12.5S complex (x-cohesinSA2), XSMC1, XSMC3, and XRAD21 associate with a different subunit, XSA2. Both XSA1 and XSA2 belong to the SA family of mammalian proteins and exhibit similarity to Scc3p, a recently identified component of yeast cohesin. In Xenopus egg extracts, x-cohesinSA1 is predominant, whereas x-cohesinSA2 constitutes only a very minor population. Human cells have a similar pair of cohesin complexes, but the SA2-type is the dominant form in somatic tissue culture cells. Immunolocalization experiments suggest that chromatin association of cohesinSA1 and cohesinSA2 may be differentially regulated. Dissociation of x-cohesinSA1 from chromatin correlates with phosphorylation of XSA1 in the cell-free extracts. Purified cdc2-cyclin B can phosphorylate XSA1 in vitro and reduce the ability of x-cohesinSA1 to bind to DNA or chromatin. These results shed light on the mechanism by which sister chromatid cohesion is partially dissolved in early mitosis, far before the onset of anaphase, in vertebrate cells.

Key words: Xenopus egg extract • sister chromatid cohesion • SMC proteins • chromosome structure • mitosis

Introduction

Sister chromatid cohesion is established along the entire length of the chromosome at the time of DNA replication and persists throughout the G2 phase (for reviews see Miyazaki and Orr-Weaver, 1994; Biggins and Murray, 1999; Nasmyth, 1999). In most organisms, each chromatid undergoes dramatic structural changes, known as condensation, at the onset of mitosis (for reviews see Koshland and Strunnikov, 1996; Hirano, 2000). The condensation process is likely to accompany a partial release of cohesion, producing two recognizable chromatids within a metaphase chromosome. Nevertheless, the linkage between the sister chromatids is maintained to ensure proper alignment of the chromosomes on the metaphase plate. The final release of cohesion takes place at the onset of anaphase, leading to complete separation of the sister chromatids. Malfunction in any one of these processes would result in chromosome missegregation and aneuploidy, potentially contributing to birth defects or tumor development (for reviews see Hassold and Jacobs, 1984; Lengauer et al., 1998).

Recent studies show that a multisubunit complex, termed cohesin, is likely to be a central player in sister chromatid cohesion. In Saccharomyces cerevisiae, Smc1p, Smc3p, and Scc1p (also known as Mcd1p) were identified in genetic screens for mutants that displayed premature separation of sister chromatids in mitosis (Michaelis et al., 1997). Scc1p/Mcd1p was also characterized as a gene product that interacts genetically and physically with Smc1p (Guacci et al., 1997). Smc1p and Smc3p belong to a large family of chromosomal ATPases (the structural maintenance of chromosomes [SMC] family), members of which are involved in many aspects of higher order chromosome architecture and dynamics (for reviews see Hirano, 1999; Strunnikov and Jessberger, 1999). In Xenopus laevis, a systematic search for SMC proteins led to the identification...
of a 14S protein complex in which XSMC1, XSMC3, and XRAD21, the homologues of Smc1p, Smc3p, and Scc1p/Mcd1p (Rad21 in _Saccharomyces cerevisiae_; Birkenbihl and Subramani, 1995), respectively, associate with two unidentified subunits, p155 and p95. Immunodepletion of Xenopus cohesin from interphase egg extracts results in the assembly of chromosomes with unpaired sister chromatids in subsequent mitosis (Losada et al., 1998). More recently, a biochemical study in _S. cerevisiae_ has further confirmed that Smc1p, Smc3p, and Scc1p/Mcd1p are present in a yeast cohesin complex, along with a new subunit called Scsp3 (Toth et al., 1999).

In _S. cerevisiae_, the cohesin complex binds to chromatin in late G1, establishes sister chromatid cohesion in the S phase, and remains bound to chromatin by metaphase (Michaels et al., 1997; Uhlmann and Nasmyth, 1998). At the metaphase-anaphase transition, the Scc1p/Mcd1p subunit is proteolytically cleaved in an Esp1p/separin-dependent manner, which in turn triggers dissociation of cohesin from chromatin and promotes sister chromatid separation (Uhlmann et al., 1999). A similar, if not identical, mechanism may also operate in S. pombe (Y anagoda, 2000). However, in vertebrate cells, the timing of the dissociation of cohesin from chromatin is drastically different from that of yeast cohesin. In X enopus, cohesin binds to chromatin during interphase, but most of it (~95%) dissociates from chromatin upon entry into mitosis, whencdc2-cyclin B gets activated (Losada et al., 1998). Immunofluorescent staining of human and mouse cells also shows that the bulk of SMC1 and SM C3 relocates from chromatin to cytoplasm in early mitosis (Schmiesing et al., 1998; Darwiche et al., 1999; Stursberg et al., 1999).

To explain these results, two models have been proposed for the role of vertebrate cohesins in sister chromatid cohesion (Losada et al., 1998). In the first model, the cohesin complex participates in holding the sister chromatids together from the S phase to G2, but it does not play a major role in cohesion from prophase to metaphase. A different protein component, such as MEI-S332 protein in *Drosophila melanogaster* (LeBlanc et al., 1999), could function as a mitosis-specific chromatid glue. In the second model, the cohesin complex is released from chromatin sequentially at two different stages of mitosis in vertebrate cells. A partial release of cohesin, and thereby a partial dissolution of cohesion, occurs in early mitosis, concomitant with condensin-mediated chromatid compaction. A small amount of cohesin that remains bound to intersister regions of the condensed chromatids may keep functioning as a glue in metaphase. The recent discovery of a pair of separin and securin molecules from X enopus and humans (Zou et al., 1999) implies that a separin-dependent cleavage of cohesin subunits (or other cohesion molecules) could trigger anaphase sister chromatid separation in vertebrates, as has been demonstrated in _S. cerevisiae_ (Uhlmann et al., 1999). Even less is known about how dissociation of cohesins from chromatin in early mitosis might be regulated in vertebrate cells. Release at this stage could also be promoted by a mechanism involving protein cleavage (Orr-Weaver, 1999). Alternatively, it could be regulated by a totally different posttranslational modification of cohesin subunits.

To address these unresolved issues, we have first identified p155, a previously uncharacterized subunit of the 14S cohesin complex, as the Xenopus orthologue of mammalian stromal antigen (SA) 1 (Carramolino et al., 1997), and named it XSA1. We have also found that a minor population of XSMC1, XSMC3, and XRAD21 associates with XSA2, a different member of the SA protein family, forming a novel cohesin complex of ~12S. Both XSA1 and XSA2 share similarity to yeast Scc3p (Toth et al., 1999). The XSA1-type (cohesin<sup>SA1</sup>) and XSA2-type (cohesin<sup>SA2</sup>) complexes are also present in human cells. Unlike in Xenopus egg extracts, however, cohesins<sup>SA2</sup> are more abundant than cohesins<sup>SA1</sup> in somatic tissue culture cells. Immunolocalization experiments have shown that cohesins<sup>SA1</sup> and cohesins<sup>SA2</sup> are differentially regulated at the level of nuclear transport in Xenopus cell-free extracts. Finally, we provide evidence that phosphorylation of the SA subunits could play a key role in dissociation of cohesins from chromatin in early mitosis.

**Materials and Methods**

**Cloning of the 155-kD Subunit of the Xenopus Cohesin Complex**

X enopus cohesin complexes were immunoprecipitated with anti-XSMC3 antibody from a Xenopus egg high speed supernatant, separated by 7.5% SDS-PAGE, and stained with Coomassie blue G. A gel fragment containing p155 was excised and digested in situ with lysylendopeptidase. The resulting peptides were fractionated by reverse phase chromatography and sequenced by Edman degradation as described previously (Bell et al., 1993). The following sequences were obtained: X(FPR)(N)(P)AE (E[A LA]; (M)YSDATLNTSQQY(VLLK); XLDSLKLK. A database search revealed that highly homologous sequences are present in a human protein called SA1 of unknown function (Carramolino et al., 1997). Taking advantage of this information, a human cDNA fragment was amplified by PCR using a 14S protein complex as a template. Oligonucleotides used for the PCR were as follows: hSA1-6, 5'-GTGGAATTCCTTTGCTGGTGCCAGCAT-G3' (EcoRI tag sequence is underlined); and hSA1-8, 5'-GCAGGATCCCTTTGCTGGTGCCAGCATG3' (BamHI tag sequence is underlined). The two primers amplified a 520-bp fragment containing a part of human SA1 cDNA (corresponding to amino acids 773-939), which was used as a hybridization probe to screen a Xenopus oocyte cDNA library (Stratagene). A filter two rounds of screening, 15 independent clones were obtained that could be classified into two groups. The major group (11/15 clones) encoded a protein most homologous to human and mouse SA1 (designated XSA1 for Xenopus SA1), whereas the minor group (4/15) encoded a protein most homologous to human and mouse SA2 (designated XSA2). A full-length sequence for XSA1 was assembled from four overlapping clones. For XSA2, a full-length sequence was determined after amplifying the 5' end sequence by nested PCR. The peptide sequences derived from microsequencing are all found in XSA1, but not in XSA2. Therefore, we conclude that the p155 cohesin subunit is identical to XSA1.

**Preparation of Antibodies**

Rabbit polyclonal antibodies were raised against synthetic peptides corresponding to the COOH-terminal amino acid sequences of XSA1 (EDDS-GFGMPMF) and XSA2 (DPASIMDESVLGVSMF). Immunization and affinity purification of antibodies were performed as described previously (Hirano et al., 1997). We also raised antisera against a recombinant XSA1 protein and obtained results similar to those with the peptide antibodies.

**Extract Preparation**

Low speed supernatants (LSSs) of X enopus eggs were prepared in XBE2 buffer (10 mM potassium-Hepes, pH 7.7, 0.1 M KCl, 2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 5 mM EGTA) as described previously (Losada et al., 1999). LSSs were further fractionated by centrifugation at 50,000 rpm at 4°C for 2 h in a Beckman TL55 rotor to yield high speed supernatants (HSSs). HSSs do
not contain membrane fractions and, thereby, cannot initiate DNA replication in vitro (Sheehan et al., 1988). Hela cells, whose nuclear extracts were prepared in buffer B (20 mM potassium-Hepes, pH 8.0, 0.1 M KC1, 2 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM β-mercaptoethanol, 20% glycerol), as described by M. Ayeda and K. Rainier (1999) with minor modifications: nuclear pellets were resuspended in 0.3 M KC1 instead of 0.2 M KC1; and a sonication step was included after homogenization of nuclei.

Immunoprecipitation, Immunodepletion, and Immunopurification

Immunoprecipitations were performed as described previously (Hirano et al., 1997). For immunodepletions, 25 μl of protein A agarose beads (Life Technologies Inc.) or a Affi-Prep protein A support (Bio-Rad Laboratories) were coated with 5 μg each of anti-XSM C1 and anti-XSM C3 antibodies, 10 μg of anti-XSA 1, 10 μg of anti-XSA 2, or 10 μg of control rabbit IgG. The antibody-coupled beads were incubated with 50 μl of extract (HSS or LSS) at 4°C for 1 h. The extent of each depletion was estimated by quantitative immunoblotting using internal standards. For affinity purification, interphase HSS was first fractionated with 35% ammonium sulfate. Most of the 9S cohesin complex remains soluble, whereas the cohesin holocomplex precipitates after incubation on ice for 30 min. The pellet was resuspended in XBE 2-gly (to half the initial volume of extract), and mixed in Microcon-30 tubes (Millipore Corp.). For the filter-binding assay, the 9S cohesin complex remains soluble, whereas the cohesin holocomplex precipitates after incubation on ice for 30 min. The pellet was resuspended in XBE 2-gly (to half the initial volume of extract), and mixed in Microcon-30 tubes (Millipore Corp.). For the filter-binding assay, the 9S cohesin complex remains soluble, whereas the cohesin holocomplex precipitates after incubation on ice for 30 min. The pellet was resuspended in XBE 2-gly (to half the initial volume of extract), and mixed in Microcon-30 tubes (Millipore Corp.).

DNA- or Chromatin-binding Assays for Purified Cofactors

The filter-binding assay (see Fig. 7 C) was performed as described previously (K. Imura et al., 1999). The binding reaction mixture (5 μl) contained 10 ng of supercoiled plasmid DNA (H/JX-1, 3.2 kb) and ~90 ng affinity-purified cofactors (phosphorylated or unphosphorylated). For the bead-binding assay (see Fig. 7 D), cofactors were mixed with three different kinds of beads: (1) beads with no coupled DNA; (2) beads with coupled DNA; and (3) beads on which interphase chromatin had been assembled in a cohesin-depleted HSS. The binding reactions (10 μl) contained 50 μg of magnetic beads (with or without 1 μg of DNA), 60 μM cofactors (phosphorylated or unphosphorylated), 1 mg/ml casein, 1 mM MgATP, and 50 mM β-glycerophosphate. 0.3 mM roscovitine was also added to the binding reactions to stop further phosphorylation by cdc2-cyclin B, which had been carried over from the previous treatment. A fter incubation at room temperature for 1 h, the beads were washed with XBE 2-0.2% NP-40, and bound proteins were analyzed by immunoblotting.

Results

The p155 Subunit of Xenopus Cohesin Is the Orthologue of Mammalian SA1

We cloned a cDNA encoding the 155-kD subunit of the X. A subunit of mammalian cohesin. The C. E. A subunit was cloned in EcoR I- and BglII-digested DNA fragments from a Xenopus cDNA library. The isolated DNA fragment was subcloned into a mammalian expression vector and transfected into cells. The transfected cells were analyzed for the expression of the 155-kD subunit by immunoblotting. The results are summarized in Table 1.

| Protein | Molecular Weight (kDa) | Expression (%) |
|---------|------------------------|----------------|
| p155    | 155                    | 80             |
| SA1      | 157                    | 70             |
| SA2      | 135                    | 60             |

The results show that the 155-kD subunit is the orthologue of mammalian SA1. The C. E. A subunit was cloned in EcoR I- and BglII-digested DNA fragments from a Xenopus cDNA library. The isolated DNA fragment was subcloned into a mammalian expression vector and transfected into cells. The transfected cells were analyzed for the expression of the 155-kD subunit by immunoblotting. The results are summarized in Table 1.
isolated another homologous cDNA that encodes the Xenopus orthologue of mammalian SA2 (termed XSA2). XSA2 is 1,194 amino acids long and has a calculated molecular mass of 137.7 kD. XSA1 has an NH$_2$-terminal extension of 78 residues that is not found in XSA2 (Fig. 1). Although the central core regions of the XSA1 and XSA2 sequences are $\sim$75% identical to each other, the COOH-terminal domain ($\sim$200 amino acids long) is considerably different between them. Consistent with our finding, Toth et al. (1999) have recently reported that Scc3p, a subunit of the Saccharomyces cerevisiae cohesin complex, shows similarity to members of the mammalian SA family.

**XSA1 and XSA2 Are Present in Different Cohesin Complexes in Xenopus Egg Extracts**

To confirm that XSA1 is indeed a cohesin subunit and to determine the relationship between XSA1 and XSA2, rabbit antibodies were prepared against synthetic peptides corresponding to their COOH-terminal sequences. By immunoblotting against a Xenopus egg extract, anti-XSA1 recognized a polypeptide of 155 kD, whereas anti-XSA2 reacted with a different polypeptide of 150 kD (data not shown). Each antibody was specific for each polypeptide and no cross-reaction was observed. We found that XSA1 and XSA2 were present in the immunoprecipitates obtained with anti-XSMC1 or anti-XSMC3 (Fig. 2, A and B, lanes 4 and 7). However, anti-XSA1 coimmunoprecipitated XSMC1, XSMC3, and XRAD21, but not XSA2 (Fig. 2 B, lane 10). Conversely, anti-XSA2 did not coimmunoprecipitate XSA1 (Fig. 2 B, lane 13). The specificity of all the immunoprecipitation reactions was demonstrated further by the addition of competing peptides (Fig. 2 B, lanes 5, 6, 8, 9, 11, 12, 14, and 15). These results indicate that XSA1 and XSA2 associate with XSMC1, XSMC3, and XRAD21 in two different complexes. Consistently, when a total egg extract was fractionated by sucrose gradient centrifugation, XSA1 and XSA2 sedimented in different positions (Fig. 2 C). The XSA1-containing complex had a sedimentation coefficient of $\sim$14S, whereas the XSA2-containing complex migrated to a position of $\sim$12.5S. The egg extract also contained a 9S heterodimer of XSMC1 and XSMC3 as reported previously (Losada et al., 1998). To assess the relative amounts of the three different forms, immunodepletion experiments were performed using anti-

---

**Figure 1.** Sequence alignment of Xenopus and human SA1 and SA2. Identical amino acid residues are shaded. These sequence data are available from GenBank/EMBL/DDBJ under accession numbers AF255017 and AF255018.
The antibodies raised against the Xenopus cohesin subunits recognize their human counterparts, allowing us to characterize the human cohesin complexes from HeLa cell extracts (Fig. 3 A). A human cohesin fraction affinity-purified using anti-XSMC3 contained hSMC1 (160 kD), hSMC3 (140 kD), and hRAD21 (125 kD). hSA1 and hSA2, the human homologues of XSA1 and XSA2, respectively, were also detected in this fraction by immunoblotting. Sucrose gradient centrifugation of a HeLa cell extract showed that most of hSMC1, hSMC3, hRAD21, hSA1, and hSA2 cosedimented in a major peak of ~135 (Fig. 3 B). As judged by immunoprecipitation, however, hSA1 and hSA2 are present in different complexes that share hSMC1, hSMC3, and hRAD21 as common subunits (Fig. 3 C). Immunodepletion experiments showed that anti-XSA1 and anti-XSA2 removed ~25 and 75%, respectively, of hSMC1, hSMC3, and hRAD21 from an extract (Fig. 3 D). Thus, in HeLa cell extracts, the ratio between the hSA1-containing complex (h-cohesinSA1) and the hSA2-containing complex (h-cohesinSA2) is about 1:3. This is in striking contrast to Xenopus egg extracts, in which the ratio between x-cohesinSA1 and x-cohesinSA2 is 10:1.

Localization of CohesinSA1 and CohesinSA2 in Xenopus Egg Extracts

Previous experiments using Xenopus cell-free extracts have shown that XSMC1, XSMC3, and XRA D21 bind to chromatin during interphase, and that most of them (~95%) dissociate from chromatin at the onset of mitosis (Losada et al., 1998). The identification of XSA1 and XSA2 as subunits of two different cohesin complexes prompted us to test whether they have different functional characters in terms of chromatin binding. First, HSSs were used to assemble interphase chromatin and single chromatid mitotic chromosomes from sperm nuclei, and they were analyzed by immunofluorescence. We found that both XSA1 and XSA2 were bound to the interphase chromatin in an indistinguishable manner, and neither of them was detectable in the condensed chromosomes (Fig. 4 A).

A n interphase LSS was used to assemble interphase nuclei with a nuclear envelope, in which a complete round of DNA replication took place. The staining of interphase nuclei with anti-XSA1 overlapped completely with the DNA staining (Fig. 4 B, a, f, and k). In contrast, anti-XSA2 primarily labeled the nuclear envelope, and there was a little sign of colocalization to chromatin inside the nucleus (Fig. 4 B, d, i, and n). We converted the reaction mixture into a mitotic state, producing mitotic chromosomes consisting of two paired sister chromatids. Weak labelling on the chromosomes was detected with anti-XSA1 (Fig. 4 B, b, g, and l), but not with anti-XSA2 (Fig. 4 B, e, j, and o). The immunofluorescent signals of anti-XSA1 were located between the sister chromatids (Fig. 4 B, c, h, and m) and often enriched on subchromosomal regions with a high tendency to bend, possibly corresponding to centromeric regions (Fig. 4 B, h and m, arrow). A though similar staining was sometimes observed with anti-XSMC3, anti-XSA1 gave us more consistent results. Immunoblotting experiments show that ~95% of XSA1 is released from chromatin upon mitotic activation (data not shown), as reported previously for XSMC1, XSMC3, and XRA D21 (Losada et al., 1998). These results suggest that a small population of x-cohesinSA1 (~5%) remains on the chromatin during metaphase, and may contribute to holding the two sister chromatids together.
The preferential association of XSA2 with the nuclear envelope could indicate that x-cohesin\(^{SA1}\) and x-cohesin\(^{SA2}\) are differentially regulated at the level of nuclear transport in this cell-free extract. To test whether this property is also common to the human complexes, affinity-purified h-cohesin\(^{SA1}\) and h-cohesin\(^{SA2}\) were introduced into Xenopus interphase L55 that had been immunodepleted of the endogenous complexes. Consistent with the behavior of XSA1 and XSA2, hSA1 localized to interphase chromatin (Fig. 4 C, a, c and e), whereas hSA2 stained the nuclear surface (Fig. 4 C, b, d and f).

**Localization of Cohesin\(^{SA1}\) and Cohesin\(^{SA2}\) in Human Tissue Culture Cells**

The localization of human cohesin complexes in HeLa cells was examined by immunofluorescence (Fig. 5). In interphase, both hSA1 and hSA2 localized to the nucleus. There was no sign of an enrichment of hSA2 on peripheral regions of the nucleus. Detergent extraction of the cells before fixation did not affect the staining of interphase cells (data not shown), suggesting that these proteins actually bind to chromatin. After nuclear envelope breakdown, the proteins were distributed throughout the whole cell, and they appeared to be excluded from the condensed chromosomes. We have been unable to detect hSA1, hSA2, or hSMC3 in mitotic chromosomes either between the sister chromatids or at centromeric regions. In telophase, hSA1 and hSA2 started to accumulate inside the newly formed daughter cell nuclei. Thus, h-cohesin\(^{SA1}\) and h-cohesin\(^{SA2}\) appear to behave very similarly throughout the cell cycle in HeLa cells: the two complexes associate with chromatin in telophase; and the bulk of them dissociate from chromatin at the onset of mitosis.

**A Cohesin Dissociation Assay Using a Solid-phase Chromatin Assembly System**

To examine how dissociation of cohesins from chromatin might be regulated in early mitosis, an in vitro assay was developed using DNA-coupled paramagnetic beads (Sandaltzopoulos and Becker, 1999). Both interphase and mitotic extracts support efficient assembly of nucleosomes around the immobilized DNA, as judged by micrococcal nuclease digestion (data not shown). The advantage of this solid-phase chromatin over sperm chromatin is that it can be easily separated from soluble components in a magnetic field. We found that cohesins, but not condensins, were recovered on chromatin assembled in an interphase extract (Fig. 6 A, lane 4). Conversely, mitotic chromatin contained condensins but not cohesins (Fig. 6 A, lane 3). Neither cohesins nor condensins were detected when uncoupled control beads were used (Fig. 6, lane 1 and 2). When immobilized chromatin containing cohesins was exposed to a mitotic extract, most cohesins dissociated and were replaced with condensins (Fig. 6 A, lane 5). These results show that this solid-phase chromatin assembly system is able to faithfully reproduce the cell cycle-specific targeting of cohesins and condensins previously described using sperm-derived chromatin (Hirano et al., 1997; Losada et al., 1998).

To test whether the mitotic dissociation of cohesins from chromatin might be accompanied by proteolytic cleavage of the XRAD21 subunit, cohesin-bound interphase chromatin was incubated with a cohesin-depleted mitotic extract (Fig. 6 A, lane 6), and the released fraction was immunoprecipitated with anti-XSM C3. The four subunits of x-cohesin\(^{SA1}\), which were released from the chromatin, remained intact, and their relative abundance was indistinguishable from that of the cohesin-bound complex (Fig. 6 A, compare lanes 4 and 7). This result suggests that dissociation of cohesins from chromatin is likely to involve a posttranscriptional modification other than proteolytic cleavage.

**Phosphorylation of Cohesins in the Cell-free Extracts**

As an initial attempt to test whether dissociation of cohesin from chromatin may be regulated by mitotic phosphorylation, we took a pharmacological approach. Interphase chro-
matin was assembled on DNA-coupled beads to allow cohesins to bind (Fig. 6 B, top, lane 1) and then okadaic acid (an inhibitor of the type 1 and type 2A phosphatases) was added into the assembly mixture. This treatment caused dissociation of cohesins from the solid-phase chromatin (Fig. 6 B, top, lane 2). The assembly interphase chromatin (I) and mitotic chromosomes (M) were fixed, isolated through a glycerol cushion, and immunostained with anti-XSA1 (e and f, green) or anti-XSA2 (g and h, green). The DNA was counterstained with DAPI (a–d, red). Merged images are also shown (i–l). The other half was driven into mitosis (M) by the addition of a mitotic LSS, incubated for another 2 h, fixed, and immunostained. A mass of entangled chromosomes (b, g, l, e, j, and o) and an individual chromosome (c, h, and m) are shown. (a–e, red) DNA; (f–h, green) anti-XSA1; (i and j, green) anti-XSA2; (k–o) merged images. Stronger staining with anti-XSA1 was often observed on a bent region of the chromosome, most likely corresponding to the pericentromeric region (arrowhead). The images of antibody staining were captured using different exposure times to visualize weak signals on the mitotic chromosomes and, therefore, the label intensities cannot be compared quantitatively. (C) Xenopus sperm nuclei were incubated in an interphase LSS depleted of x-cohesins and supplemented with h-cohesin\textsuperscript{SA1} and h-cohesin\textsuperscript{SA2}. After incubation at 22°C for 90 min, the nuclei were fixed, and hSA1 and hSA2 were detected with anti-XSA1 and anti-XSA2, respectively. (a and b, red) DNA; (c and d, green) antibody staining; (e and f) merged images. Bars, 5 μm.

Figure 4. Localization of cohesin complexes in nuclei and chromosomes assembled in Xenopus egg extracts. (A) Xenopus sperm chromatin was incubated in interphase or mitotic HSS. The assembled interphase chromatin (I) and mitotic chromosomes (M) were fixed, isolated through a glycerol cushion, and immunostained with anti-XSA1 (e and f, green) or anti-XSA2 (g and h, green). The DNA stain was also shown (i–l). (B) Xenopus sperm chromatin was incubated at 22°C for 90 min in interphase LSS, where it was replicated. Half of this interphase mixture (I) was fixed and processed for immunostaining with anti-XSA1 (a, f, and k) or anti-XSA2 (d, i, and n). The other half was driven into mitosis (M) by the addition of a mitotic LSS, incubated for another 2 h, fixed, and immunostained. A mass of entangled chromosomes (b, g, l, e, j, and o) and an individual chromosome (c, h, and m) are shown. (a–e, red) DNA; (f–h, green) anti-XSA1; (i and j, green) anti-XSA2; (k–o) merged images. Stronger staining with anti-XSA1 was often observed on a bent region of the chromosome, most likely corresponding to the pericentromeric region (arrowhead). The images of antibody staining were captured using different exposure times to visualize weak signals on the mitotic chromosomes and, therefore, the label intensities cannot be compared quantitatively. (C) Xenopus sperm nuclei were incubated in an interphase LSS depleted of x-cohesins and supplemented with h-cohesin\textsuperscript{SA1} and h-cohesin\textsuperscript{SA2}. After incubation at 22°C for 90 min, the nuclei were fixed, and hSA1 and hSA2 were detected with anti-XSA1 and anti-XSA2, respectively. (a and b, red) DNA; (c and d, green) antibody staining; (e and f) merged images. Bars, 5 μm.
Treatment of the human complexes with λ-phosphatase after reisolation from the mitotic extracts eliminated the shift (Fig. 6 C, lanes 4 and 5). Taken together, these results suggest that the SA1 and SA2 subunits become phosphorylated during mitosis, concomitant with their release from chromatin.

**Phosphorylation of Cohesins by Cdc2 In Vitro**

It has been shown that cdc2-cyclin B can directly phosphorylate condensin subunits in vitro and activate the positive supercoiling activity of the complex (Kimura et al., 1998). Similarly, cdc2-cyclin B may also phosphorylate cohesin subunits and modulate cohesin functions in vitro. When immunoprecipitated cohesins were incubated with cdc2-cyclin B in the presence of γ[32P]ATP, only XSA1 was heavily labeled (Fig. 7 A, lanes 1 and 2). Moreover, this subunit exhibited an electrophoretic mobility shift upon phosphorylation (Fig. 7 A, lanes 3 and 4). The radiolabeling and the mobility shift were both sensitive to rocospine, a CDK inhibitor (data not shown). A weak phosphorylation of XSA2 by cdc2-cyclin B could be detected, but it was difficult to study this event further because of its low abundance in the egg extracts.

One attractive possibility is that cdc2 phosphorylation of XSA1 induces dissociation of the cohesin complex from chromatin in early mitosis. To test this model, interphase chromatin was assembled in an interphase HSS, washed, and then placed in a mitotic HSS (lane 5; I > M) or a cohesin-depleted mitotic HSS (lane 6; I > ΔM). A 2-h incubation with lambda-phosphatase released the cohesin complexes from chromatin (lane 5). The fraction released from the I > ΔM chromatin beads was immunoprecipitated with anti-XSMC3 and analyzed by immunoblotting (lane 7). (B) DNA-coupled beads were incubated with an interphase HSS (I) for 1 h (lane 1). Half of the reaction mixture was incubated for another 1 h after addition of okadaic acid to 1 μM (lane 2; OA). The DNA beads were also incubated in a mitotic HSS (M) without (lane 3) or with (lane 4) 5 mM 6-dimethylaminopurine (DMAP) for 2 h. The chromatin beads were separated from the extract and washed. Cohesin fractions, which were present in chromatin (top) or extracts (middle and bottom), were detected by immunoblotting with the indicated antibodies. (C) A Xenopus interphase LSS was immunodepleted of x-cohesins using anti-XSMC3. An aliquot of the depleted extract was saved to check the absence of x-cohesins (lane 1). The remainder was divided into two parts, and one of them was converted into a mitotic state by addition of cyclin B90 (Gluzoter et al., 1991). Both LSSs depleted of x-cohesins were supplemented with purified h-cohesinSA1 and h-cohesinSA2 (~7.5 nM each). A control incubation at 22°C for 90 min, aliquots were taken from the interphase (lane 2) and the mitotic (lane 3) extracts. The human cohesin complexes were recovered from the LSS by immunoprecipitation with anti-XSMC3, and treated with buffer alone (lane 4) or λ-phosphatase (lane 5) at 30°C for 1 h. The aliquots of the extracts and the immunoprecipitates were separated by SDS-PAGE, and hSA1 and hSA2 were detected by immunoblotting with anti-XSA1 (top) and anti-XSA2 (bottom), respectively.
beads were analyzed by immunoblotting. (A) Xenopus cohesins were immunoprecipitated from an interphase HSS with anti-XSMC3, and incubated with purified cdc2-cyclin B (lanes 1 and 3) or no kinase (lanes 2 and 4) in the presence of γ[32P]ATP. A day after washing, the immunoprecipitates were separated by SDS-PAGE and analyzed by autoradiography (lanes 1 and 2) or by immunoblotting (lanes 3 and 4). (B) Solid-phase chromatin was assembled on DNA-coupled magnetic beads in an interphase HSS, washed, and treated with cdc2-cyclin B (lanes 1 and 2) or no kinase (lanes 3 and 4) in the presence of γ[32P]ATP. A day after incubation at 22°C for 1 h, proteins remaining bound to the chromatin beads (lanes 1 and 3; unreleased [U]) and those dissociated from the beads (lanes 2 and 4; released [R]) were analyzed by immunoblotting with antibodies against cohesin subunits (top) or by autoradiography (bottom). (C) Filter binding assay. Purified cohesins were first treated with no kinase (lane 1), cdc2-cyclin B (lane 3), or cdc2-cyclin B in the presence of the CDK inhibitor roscovitine (lane 4), and then incubated with plasmid DNA in a low salt buffer. Buffer alone (lane 1) or cdc2-cyclin B alone (lane 5) were also mixed with plasmid DNA. The binding reactions were passed through glass fiber filters, and washed sequentially with low salt buffer, high salt buffer, and buffer containing SDS. The ratio of bound DNA to total DNA was calculated as described previously (Kimmer et al., 1999). The average of values from three independent experiments was plotted with error bars. (D) Bead-binding assay. Unphosphorylated (lanes 1, 3, and 5) or phosphorylated (lanes 2, 4, and 6) cohesins were mixed with no-DNA beads (lanes 1 and 2; control beads), DNA-coupled beads (lanes 3 and 4; DNA beads), or solid-phase chromatin beads assembled in a cohesin-depleted interphase HSS (lanes 5 and 6; chromatin beads). A day after incubation for 1 h, the beads were washed and proteins bound to the beads were analyzed by immunoblotting.

cdc2-cyclin B. We were unable to detect cdc2-dependent dissociation of cohesin from chromatin in this purified system. A small population of cohesin was released into the soluble fraction, but this occurred even in the absence of cdc2 (Fig. 7 B, upper). Interestingly, only the soluble XSA1 in the released fraction, but not chromatin-bound XSA1, was phosphorylated by cdc2 under those conditions (Fig. 7 B, lower). This observation led to another possibility that phosphorylation of XSA1 by cdc2 might prevent reassociation of cohesins once they are released from chromatin during early mitosis. To investigate this, a filter-binding assay was first performed using affinity-purified Xenopus cohesins and naked DNA as a substrate. We found that cohesins can bind directly to DNA in this assay (Fig. 7 C, lane 2). Pretreatment of the cohesin fraction with cdc2-cyclin B significantly reduced its DNA-binding activity (Fig. 7 C, lane 3), and this effect was reversed when roscovitine was added into the phosphorylation reaction (Fig. 7 C, lane 4). In a complementary assay, DNA-coupled magnetic beads were used as binding substrates. Purified cohesins were found to efficiently bind to the DNA-coupled beads (Fig. 7 D, lane 3) but not to uncoupled control beads (Fig. 7 D, lanes 1 and 2). The amount of cohesins bound to the DNA beads was drastically reduced when cohesins were prephosphorylated with cdc2-cyclin B (Fig. 7 D, lane 4). Finally, we tested the binding of cohesins to the solid-phase chromatin assembled on the DNA-coupled beads. A gain, the unphosphorylated form of cohesins exhibited a higher affinity to chromatin compared with the phosphorylated form (Fig. 7 D, lanes 5 and 6). These results show that phosphorylation of the XSA1 subunit by cdc2 decreases binding of cohesins to DNA and chromatin in vitro.

Discussion

Two Different Cohesin Complexes in Vertebrate Cells

Cohesins are multisubunit protein complexes that play a major role in sister chromatid cohesion. In this paper, we report the identification and characterization of two new cohesin subunits from Xenopus egg and human cell extracts. Both of them belong to the mammalian SA protein family, whose cellular functions were previously unknown (Carramolino et al., 1997). In Xenopus egg extracts, XSA1 and XSA2 associate with XSMC1, XSMC3, and XRAD21 to form a 14S complex (x-cohesins\textsuperscript{SA1}) and a 12.5S complex (x-cohesins\textsuperscript{SA2}), respectively. The difference between the sedimentation coefficients of the two complexes could be explained if x-cohesin\textsuperscript{SA1} had an additional subunit. In fact, our previous study showed that a 95-kD polypeptide (p95), whose identity remains to be determined, cosediments with the 14S complex by sucrose gradient centrifugation (Losada et al., 1998). The low abundance of x-cohesins\textsuperscript{SA2} (1/10 of x-cohesins\textsuperscript{SA1}) explains why this population has been overlooked before. HelA cell extracts also contain the SA1- and SA2-type cohesin complexes (h-cohesins\textsuperscript{SA1} and h-cohesins\textsuperscript{SA2}, respectively). The two complexes share human homologues of XSMC1, XSMC3 (Schmiesing et al., 1998), and XRAD21 (McKay et al., 1996) as common subunits, and have similar sedimentation coefficients of ~13S. There is no evidence for the presence of p95 in h-cohesins\textsuperscript{SA1}, which is consistent with its S value being smaller than that of x-cohesins\textsuperscript{SA1}. The ratio between cohesins\textsuperscript{SA1} and cohesins\textsuperscript{SA2} is 1:1 in Xenopus egg extracts, and 1:3 in HelA cell extracts. The dominance of the SA1-type complex in Xenopus egg is likely to be characteristic of early embryonic systems, because the SA1:SA2 2 ratio in Xenopus somatic tissue culture cells XL177 is very similar to that observed in HelA cells (data not shown).

Recently, Toth et al. (1999) reported that Scs3p, a gene product required for sister chromatid cohesion in S. cerevisiae, has similarity to mammalian SA proteins, and is a
subunit of the yeast cohesin complex containing Smc1p, Smc3p, and Scc1p/Mcd1p/Rad21. Thus, the subunit composition of the cohesin complexes is highly conserved from yeast to humans. Since Scc3p is the only SA-like sequence present in the genome of S. cerevisiae, it is most likely that a single cohesin complex functions in the mitotic cycle in this organism.

**Chromatin Association of CohesinSA1 and CohesinSA2**

Are there any functional differences between the SA 1-type and SA 2-type complexes? In nuclei assembled in an LSS, which are surrounded by a nuclear membrane, x-cohesin\textsuperscript{SA1} and x-cohesin\textsuperscript{SA2} exhibit different localization. X SA 1 localizes to interphase chromatin within the nuclei, whereas X SA 2 is apparently present on the nuclear envelope. Interestingly, when purified human cohesins are introduced into a X enopus LSS, hSA 1 and hSA 2 behave like XSA 1 and XSA 2, respectively. These observations suggest that cohesin\textsuperscript{SA1} and cohesin\textsuperscript{SA2} are differentially regulated at the level of nuclear transport in this cell-free system. The p95 subunit present in x-cohesin\textsuperscript{SA1} could play an active role in this process. When added into an X enopus egg extract, only h-cohesin\textsuperscript{SA1}, but not h-cohesin\textsuperscript{SA2}, may recruit p95 and thereby form a transport-competent complex. The NH\textsubscript{2}-terminal tail sequence conserved among SA 1 proteins could offer a binding site for p95. The rapid transport of the abundant SA 1-type complex across the nuclear membrane may be a strategy unique to the early embryonic cell cycle. It is known that, during early development of X enopus, a nuclear envelope assembles around each individual chromosome shortly after anaphase (forming the so-called karyomeres) to allow rapid initiation of DNA replication before full reconstitution of the nucleus (Lemaître et al., 1998). Later in development, a slower reformarnation of the nuclear membrane may provide enough time for cohesins to directly bind to the chromatin at the end of mitosis. In fact, no significant differences were observed in the timing of association of SA 1 and SA 2 with chromatin in human or X enopus somatic tissue culture cells, as judged by immunofluorescent staining (Fig. 5 and data not shown). Consistently, if nuclear envelope assembly is suppressed by using HSS, both X SA 1 and X SA 2 can associate with interphase chromatin with similar efficiency in the X enopus cell-free system (Fig. 4 A).

It is formally possible that cohesin\textsuperscript{SA1} and cohesin\textsuperscript{SA2} have distinct properties in terms of chromatin binding. A recent study in S. cerevisiae has shown that a protein complex containing Scc2p and Scc4p is required to load cohesin onto chromatin (Ciosk et al., 2000). In vertebrate cells, specific loading factors could exist for the two different cohesin complexes and determine their specialized functions by recruiting them to different chromosomal loci.

**Do Cohesins Dissociate from Chromatin in Two Steps in Vertebrate Cells?**

In S. cerevisiae, dissociation of cohesin from chromatin in anaphase leads to a single-step separation of sister chromatids (Uhlmann et al., 1999). However, in vertebrate cells, dissolution of sister chromatid linkage may be regulated by a two-step mechanism (Hirano, 2000). The first step initiates at the onset of mitosis when tight cohesion between interphase chromatids is partially released, allowing each chromatid to condense. The second step occurs at the metaphase-anaphase transition and results in complete separation of the sister chromatids. Our current results suggest that dissociation of cohesins from chromatin may accompany the two-step resolution of cohesion in vertebrate cells. When the nuclei that are assembled in X enopus LSS are converted into chromosomes consisting of two paired sister chromatids, most X SA 1 and X SA 2 dissociate from the nuclear structures, a result consistent with previous analysis of the other cohesin subunits (Losada et al., 1998). A small population of X SA 1, however, is detectable between the two sister chromatids, suggesting that a small amount of cohesins could, in fact, play a role in holding the sister chromatids in metaphase. The apparent enrichment of x-cohesin\textsuperscript{SA1} in centromeric regions is consistent with the recent identification of cohesin association sites in S. cerevisiae by chromatin immunoprecipitation experiments (Blat and Kleckner, 1999; Megee et al., 1999; Tanaka et al., 1999). However, it should be added that we have been unable to detect any of the cohesin subunits on metaphase chromosomes in tissue culture cells. The interstitial signals may be very sensitive to staining conditions and difficult to detect on chromosomes assembled in the cell. Alternatively, a cohesion molecule other than cohesin could play a predominant role in holding sister chromatids together in somatic metaphase chromosomes.

**Regulation of Cohesin by Mitotic Phosphorylation In Vitro**

If cohesins are released from chromatin at two different stages of mitosis, how are they regulated differentially? A recent study shows that vertebrate cells have a pair of separin and securin molecules, which may play a role in anaphase sister chromatid separation (Zou et al., 1999). However, it remains to be determined, whether separin-mediated cleavage of the RAD21 subunit triggers the release of cohesin at the onset of anaphase in vertebrate cells, as has been shown in S. cerevisiae (Uhlmann et al., 1999).

The current study focuses on the mechanism that promotes dissociation of cohesin from chromatin in early mitosis, and provides evidence that it may involve protein phosphorylation. We have developed an in vitro assay using DNA-coupled paramagnetic beads that faithfully recapitulates this process. Binding of cohesins to the solid-phase chromatin is interphase-specific. Dissociation of cohesins from chromatin can be induced by adding a phosphatase inhibitor into the assembly mixture, or by exposing purified interphase chromatin to a mitotic extract. X RAD21 is not proteolytically cleaved under this condition. Purifiedcdc2-cyclin B phosphorylates cohesin in its soluble form, but not in its chromatin-bound form. We have also found that purified x-cohesin can directly bind to DNA and chromatin, and that phosphorylation by cdc2-cyclin B in vitro reduces the affinity of the complex for both substrates. On the basis of these results, two scenarios can be considered for the possible role of cdc2-mediated phosphorylation of cohesins. First, cdc2 could directly phosphorylate cohesin subunits, and thereby actively induce release of the com-
plex from chromatin in early mitosis. Our inability to reconstitute this reaction in vitro using purified chromatin and cdc2-cyclin B could be explained if additional factors were required to make the chromatin-bound form of cohesion accessible to phosphorylation by cdc2. Second, a mitotically activated factor other than cdc2 could play a primary role in the dissociation of cohesion. Cdc2-dependent phosphorylation then could contribute to preventing the released cohesins from reassociating with chromatin.

X SA 1 is the major phosphate acceptor both in the cell-free extracts and in the cdc2 phosphorylation reaction. The low abundance of x-cohesin^SA2^2 has hindered similar studies with XSA2. Nevertheless, both human SA 1 and SA 2 are phosphorylated when they are introduced in Xenopus egg extracts, suggesting that cohesin^SA1^ and cohesin^SA2^ may be regulated by a similar phosphorylation process. It is currently unknown whether Scc3p is phosphorylated in S. cerevisiae. On the other hand, Rad21 is phosphorylated in a cell-specific manner in S. pombe (Birkenbihl and Subramani, 1995), but the functional impact of this modification is not clear. In contrast, there is no evidence for phosphorylation of the X RAD 21 subunit in Xenopus cohesin. It is reasonable to speculate that different organisms use a different repertoire of regulation. For example, the activities of the X enopos condensin complex are modulated by phosphorylation of its non-SMC subunits (Kimura et al., 1998), whereas the equivalent complex in S. pombe is regulated at the level of nuclear transport by phosphorylation of one of its SM C subunits (Sutani et al., 1999).

**Dynamic Exchange of Non-SMC Subunits in Mitotic and Meiotic Cohesins**

Emerging lines of evidence suggest that the SM C subunits can associate with a variety of non-SM C partners, thereby fulfilling unique functions at different developmental stages of an organism or acquiring new functions during evolution (Hirano, 1999; Strunnikov and Jessberger, 1999). The current finding of two different SA subunits in vertebrate cohesins offers additional support to this notion. Even more drastic examples of the exchange of non-SM C partners recently have been inferred from studies on mitotic chromosome dynamics. Rec8p is a meiosis-specific SMC partners recently have been inferred from studies on meiosis.

This work was supported by grants from the Human Frontier Science Program and the Pew Scholars Program in the Biomedical Sciences (to T. Hirano) and the National Institutes of Health (to R. Kobayashi). A. Losada was the recipient of a fellowship from the Robertson Research Fund.

Submitted: 24 April 2000
R evised: 13 June 2000
Accepted: 14 June 2000

**References**

Belli, S.P., R. Kobayashi, and B. Stillman. 1993. Yeast origin recognition complex functions in transcription silencing and DNA replication. Science. 262: 1844-1849.

Biggins, S., and A. Murray. 1999. Sister chromatid cohesion in mitosis. Curr. Opin. Cell Biol. 9:230–236.

Birkenbihl, R.P., and S. Subramani. 1995. The rad21 gene product of Schizosaccharomyces pombe is a nuclear, cell cycle-regulated phosphoprotein. J. Biol. Chem. 270:7703–7711.

Blat, Y., and N. Kleckner. 1999. Cohesins bind to preferential sites along yeast chromosome III, with differential regulation along arms versus the centric region. Cell. 98:249–259.

Carramolino, L., B.C. Lee, A. Zagallo, A. Peled, J. Barthelemy, Y. Shav-Tal, I. Prieto, P. Carmi, Y. Gochev, G. Gonzalez de Buitrago, et al. 1997. SA I, a nuclear protein encoded by one member of a novel gene family: molecular cloning and detection in hemopoietic organs. Gene. 193:151–159.

Ciepek, R., M. Shirayama, A. Shevchenko, T. Tanaka, A. Toth, A. Shevchenko, and K. Nasmyth. 2000. Cohesins binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol. Cell. 5:243–254.

Darwiche, N., L.A. Freeman, and A. Strunnikov. 1999. Characterization of the components of the putative mammalian sister chromatid cohesion complex. Gene. 233:99–47.
