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Characterization of Vertebrate Cohesin Complexes and Their Regulation in Prophase

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Abstract. In eukaryotes, sister chromatids remain connected from the time of their synthesis until they are separated in anaphase. This cohesion depends on a complex of proteins called cohesins. In budding yeast, the anaphase-promoting complex (APC) pathway initiates anaphase by removing cohesins from chromosomes. In vertebrates, cohesins dissociate from chromosomes already in prophase. To study their mitotic regulation we have purified two 14S cohesin complexes from human cells. Both complexes contain SMC1, SMC3, SCC1, and either one of the yeast Scc3p orthologs SA1 and SA2. SA1 is also a subunit of 14S cohesin in *Xenopus*. These complexes interact with PDS5, a protein whose fungal orthologs have been implicated in chromosome cohesion, condensation, and recombination. The bulk of SA1- and SA2-containing complexes and PDS5 are chromatin-associated until they become soluble from prophase to telophase. Reconstitution of this process in mitotic *Xenopus* extracts shows that cohesin dissociation does neither depend on cyclin B proteolysis nor on the presence of the APC. Cohesins can also dissociate from chromatin in the absence of cyclin-dependent kinase 1 activity. These results suggest that vertebrate cohesins are regulated by a novel prophase pathway which is distinct from the APC pathway that controls cohesins in yeast.

Key words: anaphase-promoting complex • cell cycle • chromosome • mitosis • sister chromatid cohesion

Introduction

In eukaryotic cells, duplicated DNA molecules ("sisters") remain physically connected by cohesion from the time of their synthesis in S phase until they are separated in anaphase. Cohesion is a prerequisite for the bipolar attachment of chromatid pairs to the spindle apparatus in mitosis. Sister chromatid cohesion therefore enables the equal segregation of the duplicated genome to forming daughter cells long after DNA replication has occurred.

To initiate anaphase, sister chromatid cohesion has to be dissolved. In presumably all eukaryotes, this event depends on activation of the anaphase-promoting complex (APC),[1] a cell cycle–regulated ubiquitin–protein ligase that targets proteins for destruction by the 26S proteasome (reviewed by Morgan, 1999; Peters, 1999). In budding yeast, the APC initiates anaphase by activating a pathway that removes chromosomal proteins from sister chromatids. These proteins are called cohesins because they are required for sister chromatid cohesion in both yeast and *Xenopus*. Although it is not yet known whether cohesins directly connect sister chromatids or mediate cohesion indirectly, it is tempting to speculate that the APC-dependent removal of cohesins liberates sisters for poleward movement and thereby initiates anaphase (reviewed by Koshland and Guacci, 2000; Nasmyth et al., 2000).

In budding yeast and *Xenopus*, four or five cohesin proteins form a 14S cohesin complex (Losada et al., 1998; Toth et al., 1999). The yeast complex contains the subunits Smc1p, Smc3p, Scc1p/Mcd1p, and Scc3p (Strunnikov et al., 1993; Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999). Smc1p and Smc3p belong to the SMC protein family, which is represented in bacteria and eukaryotes. Members of this family were first identified in scaffold preparations of metaphase chromosomes (Lewis and Laemmli, 1982) and are now known to be required for a variety of chromosomal functions, including cohesion, condensation, recombination, and gene dosage compensation (reviewed in Jessberger et al., 1998; Strunnikov, 1998; Hirano, 1999). In budding yeast, all four cohesin subunits are associated with chromatin from S phase until the onset of anaphase (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999), consistent with the notion that the binding of the cohesin complex to replicated DNA is required for the establishment and maintenance of cohesion.

A number of additional proteins have been shown to have a role in cohesion, some possibly by interacting with 14S cohesin. Experiments in budding yeast indicate that

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[1]*Abbreviations used in this paper:* APC, anaphase-promoting complex; CDK1, cyclin-dependent kinase 1; DAPI, 4′, 6′-diamidino-2-phenylindole.

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the binding of 14S cohesin to DNA in S phase requires Scc2p and Scc4p (Ciosk et al., 2000) and that the establishment of cohesion in S phase depends on Eco1p/Ctf7p (Skibbens et al., 1999; Toth et al., 1999). However, these proteins are not essential for the maintenance of cohesion in G2 and are not part of 14S cohesin. In fission yeast, the Scc2p ortholog Mis4p and the Eco1p/Ctf7p-related protein Eso1p are also required for cohesion (Furuya et al., 1998; Tanaka et al., 2000). In *Aspergillus*, genetic interactions between BIMD and the Smc3p ortholog SUDA suggest that BIMD may physically or functionally interact with 14S cohesin (Holt and May, 1996). This notion is consistent with the observation that BIMD orthologs in *Sordaria* and budding yeast, called Spo76p and Pds5p, respectively, are required for chromosome cohesion and condensation (van Heemst et al., 1999; Hartman et al., 2000; Panizza, S., and K. Nasmyth, personal communication). In *Drosophila*, MEI-S332 and ORD have important roles in meiotic cohesion, but if and how these proteins interact with 14S cohesin is not yet known (Kerrebrock et al., 1992; Bickel et al., 1996; Moore et al., 1998).

An essential role of 14S cohesin subunits in sister chromatid cohesion was first suggested by experiments that showed that budding yeast *smc1*, *smc3*, *scc1/mcd1*, and *scc3* mutants are able to separate sister chromatids in the absence of APC activity (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999). These results and the observation that cohesin subunits dissociate from chromatin at the onset of anaphase suggested that APC activation initiates anaphase by removing 14S cohesin from chromosomes. The APC mediates this event by ubiquitinating Pds1p (Cohen-Fix et al., 1996), a protein that binds and apparently inhibits the anaphase activator Esp1p (Ciosk et al., 1998). After ubiquitin-dependent proteolysis of Pds1p, Esp1p cleaves Scc1p/Mcd1p and thereby mediates the dissociation of 14S cohesin from chromosomes (Uhlmann et al., 1999, 2000). To illustrate its activating role in the separation of sister chromatids, Esp1p and its orthologs in other eukaryotes are now called separins or separases, whereas Pds1p and its orthologs are called securins (Nasmyth et al., 2000; Uhlmann et al., 2000; Waizenegger et al., 2000; Yanagida, 2000).

Several observations indicate that the APC–separase pathway is not only essential for anaphase in yeast but also in other eukaryotes. For example, the separins Cut1p and BIMB are required for anaphase in fission yeast and *Aspergillus* (May et al., 1992; Funabiki et al., 1996b), respectively, and APC-dependent proteolysis of the securins Cut2p and PTTG is essential for sister chromatid separation in fission yeast and *Xenopus* (Funabiki et al., 1996a; Zou et al., 1999). Furthermore, a large body of evidence suggests that the APC and its mitotic activator CDC20/Fizzy are required for sister chromatid separation in all eukaryotes, including *Drosophila* and vertebrates (reviewed by Peters, 1999). The notion that the APC–separase pathway may control anaphase is furthermore consistent with the evolutionary conservation of cohesins. *Xenopus* 14S cohesin contains orthologs of yeast Smc1p, Smc3p, and Scc1p/Mdc1p (called XRad21) and two unknown proteins of 155 and 95 kD (Losada et al., 1998). Immunodepletion experiments demonstrated that this complex is required for proper sister chromatid cohesion. Despite these similarities with the yeast complex, *Xenopus* 14S cohesin has been shown to dissociate from chromosomes already in prophase, i.e., long before sisters separate and before the APC is thought to be activated. Similar observation have been made in mouse cells (Darwiche et al., 1999). In vertebrates, it is therefore not known whether the mitotic dissociation of 14S cohesin from chromosomes depends on the APC–separase pathway, as it does in yeast, and how sister chromatid cohesion is maintained between prophase and the onset of anaphase.

To address these questions we have further characterized cohesin complexes in *Xenopus* and humans and begun to study their mitotic regulation. We show that two distinct 14S cohesin complexes exist in human somatic cells, each containing SMC1, SMC3, SCC1, and either one of two Scc3p homologues, called SA1 and SA2. SA1 is also a subunit of 14S cohesin in *Xenopus*. Both human and *Xenopus* cohesin complexes bind to PDS5, an ortholog of *Aspergillus* BIMD, *Sordaria* Spo76p, and budding yeast Pds5p. The bulk of both SA1- and SA2-containing complexes and PDS5 dissociates from condensing chromatin in late prophase and rebind in telophase. In *Xenopus* extracts, the mitosis-specific dissociation of cohesin complexes from chromatin does neither depend on cyclin B proteolysis nor on the presence of the APC, suggesting that activation of the APC–separase pathway is not required for this event. Also cyclin-dependent kinase 1 (CDK1) activity is not essential for the mitotic solubilization of cohesin complexes. We therefore propose that a novel prophase pathway regulates the dissociation of 14S cohesin from chromatin in vertebrates which is distinct from the APC–separase pathway that regulates cohesins in yeast.

Materials and Methods

cDNA Clones

cDNAs were provided by: Dov Zipori (The Weizmann Institute of Science, Rehovot, Israel) (human SA1 and SA2; Carramolino et al., 1997); Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan) (human SCC1/KIAA0078; Nomura et al., 1994; human SMC1/KIAA0178; and partial human PDS5/KIAA0648; Ishikawa et al., 1998); Yoshimi Takai (Osaka University Graduate School of Medicine/Faculty of Medicine, Suita, Japan) (human SMC3/HCAP; Shimizu et al., 1998). For the expression of cDNAs as [^{35}S]methionine-labeled proteins coupled transcription–translation reactions in rabbit reticulocyte lysates (Promega) were used.

Antibodies

SA1, SA2, and PDS5 peptide antibodies were raised in rabbits. To allow covalent coupling to keyhole limpet hemocyanin a Cys residue was added to the NH_2 terminus of all peptides. The following peptides were used: human SA1, C-KRKRGRPGRPPSTNKKPRKS (antibody 444) and C-SSSSKTSSVRNKKGRPPLHKKR (antibody 445); human SA2, C-SSRGSTVRSKKSKPSTGKRKVV (antibody 446) and C-DLPPSKNRRERTELKPDFFD (antibody 447); human PDS5, C-PRRGRRPKSESQGNATKND; *Xenopus* PDS5, C-NATGRRPYSRSTGSEISNNVSINSES (antibody 647) and C-GAQEAANAKVPKQDSTAKKTAQRPIDLHR (antibody 648). All rabbit antibodies were affinity purified. Polyclonal mouse antibodies were raised against peptides corresponding to sequences of *Xenopus* XCAP-E/SMC2 (C-SKTKERRNRMEVDK), XCAP-C/SMC4 (C-AAKGLAEMQSVGCA), *Xenopus* SMC1 (C-DLTKYPDANPNPND), human SMC3 (C-EMAKDFVEDDTTHG), *Xenopus* SMC3 (C-EQAKDFVEDDTTHG), and the human PDS5 peptide described above. Additional antibodies were kindly provided by Rolf Jessberger (Mount Sinai School of Medicine, New York, NY; anti-SMC1 and SMC3), Christine Michaelis and Irene Waizenegger (Research Institute of Molecular Pathology, Vienna, Austria; anti-SCC1), Laura Lederer and Peter Jackson (Stanford Medical

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School, Stanford, CA; anti-*Xenopus* MCM3), Tim Hunt (Imperial Cancer Research Fund, Herts, UK; anti-*Xenopus* CDK1, cyclin A and B), and Ulrich Laemmli (University of Geneva, Geneva, Switzerland) and Daniel Bogenhagen (State University of New York, Stony Brook, NY; anti-*Xenopus* topoisomerase II). Antibodies to human topoisomerase II were from Boehringer, antibodies to human CDK1 from Santa Cruz Biotechnology, Inc., and antibodies specific for histone H3 phosphorylated on serine 10 were from Upstate Biotechnology. Proteasome antibodies (Peters et al., 1994) and CDC27 antibodies (Kramer et al., 1998) have been described previously.

Protein Fractionation, Immunoprecipitation, and Immunoblotting

Crude *Xenopus* interphase egg extracts were prepared as described (Vorlaufer and Peters, 1998) except that fresh extracts were used in some experiments. Extracts from cultured HeLa cells were prepared in immunoprecipitation buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.2% Nonidet P-40, 20 mM β-glycerophosphate, 10% glycerol, 1 mM NaF, 0.5 mM DTT) using a Potter-Elvejem glass-Teflon homogenizer. 4 ml buffer were used per 2×10^8 cells. Crude lysates containing 10 mg protein/ml were stored at −70°C. Density gradient centrifugation of HeLa 100,000 *g* fractions, *Xenopus* interphase egg extract 100,000 *g* fractions, or purified cohesin complexes was done using 5–30% sucrose gradients, which were centrifuged for 18 h at 36,000 rpm in a SW40 rotor (Beckman Coulter) at 4°C. Gradients were prepared with a Biocom gradient master and 400 μl fractions were collected with a density gradient fractionator (ISCO).

For immunoprecipitation experiments, affinity-purified antibodies were coupled to Affiprep protein A beads (Bio-Rad Laboratories) in a ratio of 1 mg antibodies to 1 ml beads. The antibody beads were rotated over-end in HeLa or *Xenopus* extracts for 90 min at 4°C. A ratio of 10 μl beads to 1–2 mg of protein in the extract was used. Subsequently, the beads were washed four times with immunoprecipitation buffer and bound proteins were eluted with 100 mM glycine-HCl, pH 2.0. *Xenopus* PDS5 immunoprecipitates were washed with XB (100 μm KCl, 1 μM $MgCl_2$, 0.1 μM $CaCl_2$, 20 μM Hepes, pH 7.7) plus 150 mM KCl and 0.2% NP-40 (Vorlaufer and Peters, 1998). For the immunopurification of 14S cohesin, 447 antibody beads were incubated with extracts (1–2 mg total protein per 10 μl beads) and the beads were washed several times with immunoprecipitation buffer plus 500 mM NaCl. Bound proteins were eluted either by low pH as above or by incubating the beads for 1 h at 4°C in three volumes of a solution containing 1 mg/ml 447 peptide and 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.5 mM DTT, 0.02% Tween-20. Samples were analyzed by SDS-PAGE followed by silver staining or immunoblotting as described (Gieffers et al., 1999).

Immunofluorescense Microscopy

Cultured cells grown on coverslips were fixed for 10 min at room temperature with 4% paraformaldehyde in PBS and then incubated for 5 min with 50 mM NH_4Cl. Specimens were subsequently washed with PBS, permeabilized for 10 min with PBS plus 0.1% Triton X-100, and incubated for 20 min with antibodies diluted in PBS plus 1% BSA. Secondary antibodies were labeled with FITC or Cy3 (Sigma-Aldrich). Specimens were embedded in Moviol 4-88 (Hoechst Pharmaceuticals) supplemented with 1 μg/ml 4′, 6′-diamidino-2-phenylindole (DAPI).

Cohesin Chromatin Binding and Dissociation Assays

To monitor the dissociation of cohesins from HeLa chromatin in *Xenopus* egg extracts, 10 μl of crude HeLa lysate corresponding to 100 μg protein was centrifuged for 20 min at 4°C at 13,000 rpm in a microcentrifuge and the supernatant was removed. 35 or 40 μl of interphase *Xenopus* egg extract was added to the chromatin-enriched pellet, gently mixed, and incubated at room temperature for various periods of time. For kinetic experiments, either larger reaction mixtures were used or multiple reactions were started in parallel and stopped after different incubation periods. To monitor the cell cycle state of the extracts, in vitro translation mixtures containing ^{35}S-labeled cyclin B and CDC25 were each added in a 1:20 dilution. 10 ng/μl recombinant purified sea urchin cyclin B Δ90 was added to drive extracts into mitosis. In some experiments the extracts were stimulated to enter a mitotic state by cyclin B Δ90 and stabilized in this state by addition of 1 μM okadaic acid (Calbiochem-Novabiochem) dissolved in DMSO before they were incubated with chromatin. The reactions were terminated by dilution of the sample in 160 μl of ice-cold XB buffer and 50 μM sucrose containing 0.25% Triton X-100 (XB2 buffer). To reisolate chromatin the samples were centrifuged in 1.5 ml microcentrifuge tubes through 1 ml density cushions (1 M sucrose in XB2) for 30 min at 4°C and 12,500 rpm (8,000 *g*) in a HB-6 rotor (Beckman Coulter). In some experiments samples were centrifuged for 30 min at 4 °C and 30,000 rpm in ultraclear 5 × 41 mm centrifuge tubes filled with 400 μl sucrose buffer, using a SW50.1 rotor (Beckman Coulter). The supernatant was removed and the chromatin pellets were mixed with SDS sample buffer and analyzed by SDS-PAGE and immunoblotting.

To generate *Xenopus* fractions defective in cyclin B proteolysis, interphase extracts were centrifuged for 1 h at 100,000 *g* and 4°C. Chromatin was assembled by addition of 3,200 demembranated sperm nuclei (Murray, 1991) per μl of 100, 000 *g* supernatant fraction and incubation for 60 min at room temperature. To immunodeplete the APC, affinity-purified CDC27 antibodies or control antibodies were covalently coupled to Affiprep protein A beads. 100 μl *Xenopus* interphase extract was incubated with 20 μl antibody beads for 1 h on ice. After removal of the beads chromatin was assembled by addition of sperm nuclei as above. 20 μl aliquots were removed, diluted with 180 μl XB2 buffer containing 0.25% Triton X-100, and analyzed as above.

To inhibit CDK1, 0.8 mM roscovitine (Calbiochem-Novabiochem) dissolved in DMSO was added to mitotic extracts and total histone H1 kinase activity was measured as described (Vorlaufer and Peters, 1998). To generate extracts devoid of mitotic cyclins, laid *Xenopus* eggs were treated for 15 min with 100 μg/ml cycloheximide. Meiotic exit was then triggered in the continued presence of 20 μg/ml cycloheximide by ionophore addition and extracts were prepared 45 min later. After chromatin assembly from sperm nuclei 1 μM okadaic acid was added to stimulate entry into a pseudo-mitotic state and samples were analyzed as above.

To generate cultured cells devoid of mitotic cyclins human diploid fibroblasts were grown to confluency. Cells were then treated simultaneously with 10 μg/ml cycloheximide and either with 1 μM okadaic acid or DMSO. After 2.5 h cells were harvested and homogenized as described for HeLa cells above. Chromatin fractions were isolated by spinning 40 μl crude lysate through 1 M sucrose cushions.

Results

Identification of the Scc3 Homologues SA1 and SA2 as Subunits of Two Distinct Human 14S Cohesin Complexes

The Scc3p subunit of budding yeast cohesin complexes is homologous to a family of closely related mammalian nuclear proteins called stromal antigens (Toth et al., 1999). Mouse and human cDNAs encoding two different stromal antigens (SA1–SA2) have been described (Carramolino et al., 1997), but the function of these proteins is unknown. To test whether they are cohesin subunits, we generated a panel of antibodies against human SA1 and SA2. In immunoblot experiments, the antibody 444 raised against a peptide of SA1 reacted specifically with in vitro–translated SA1 and with a 150-kD protein of similar electrophoretic mobility in extracts from HeLa cells and from *Xenopus* eggs, but not with in vitro–translated SA2 (Fig. 1 A). Antibody 446 raised against a peptide of SA2 reacted specifically with the human 140-kD SA2 protein (Fig. 1 A), whereas two other antibodies (445 and 447) recognized both SA1 and SA2 (data not shown).

When we separated HeLa cell extracts by anion exchange and gel filtration chromatography, both SA1 and SA2 cofractionated with the known subunits of 14S cohesin, SMC1, SMC3, and SCC1 (data not shown). In density gradient centrifugation experiments, SMC1 and SMC3 sedimented as 9S and 14S cohesin complexes, as previously shown for their *Xenopus* orthologs (Losada et al., 1998). SA2 cosedimented entirely and SA1 in part with SMC1, SMC3, and SCC1 (Fig. 1 B), suggesting that they may be subunits of 14S cohesin. To test this hypothesis we performed coimmunoprecipitation experiments that were

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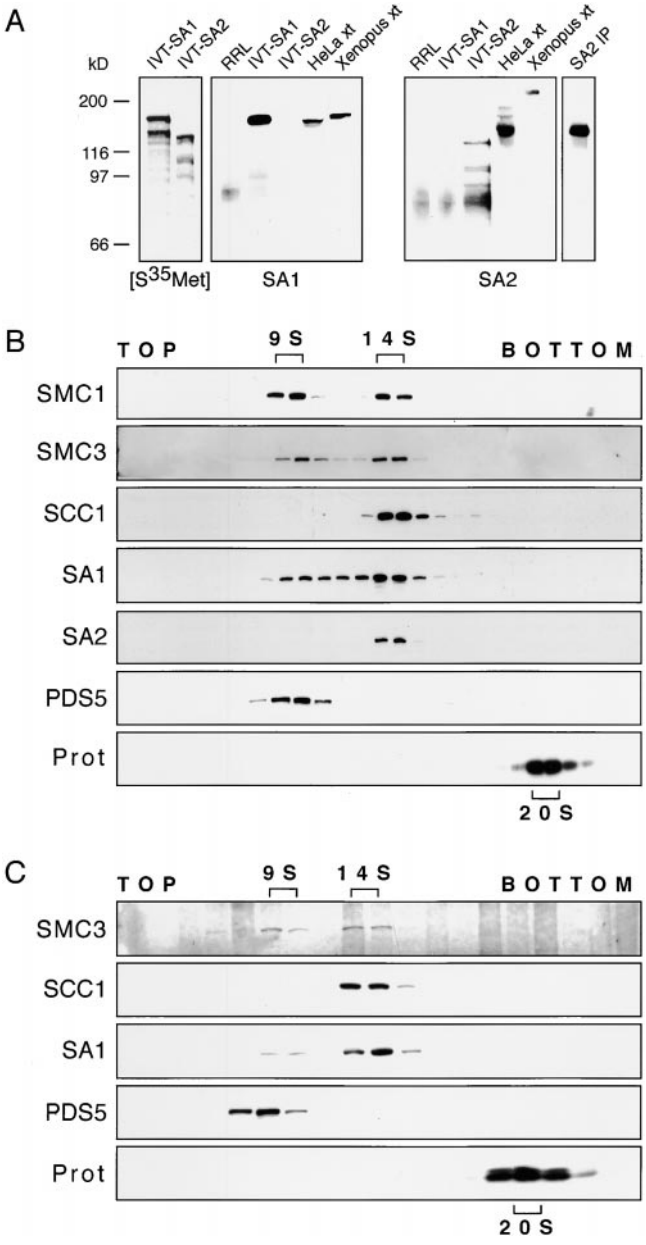

Figure 1. Fractionation of human cohesin complexes by sucrose density gradient centrifugation. (A) Characterization of SA1 and SA2 antibodies. (Left) PhosphorImager scan of in vitro–translated ^{35}S-labeled human SA1 and SA2 (IVT-SA1, IVT-SA2) separated by SDS-PAGE. Other panels, control rabbit reticulocyte lysate (RRL), in vitro–translated SA1 and SA2, protein extracts (xt) from HeLa cells, and *Xenopus* interphase egg extracts, and SA2 (446) immunoprecipitates isolated from HeLa extracts (SA2 IP) were analyzed by SDS-PAGE and immunoblotting with specific SA1 (444) or SA2 (446) antibodies. (B) Sucrose gradient fractions containing proteins from logarithmically growing HeLa cells were analyzed by SDS-PAGE and immunoblotting with antibodies to the indicated proteins. SA1 and SA2 were detected with antibodies 444 and 446, respectively. Prot, proteasome. (C) Sucrose gradient fractions containing proteins from *Xenopus* interphase extract were analyzed by SDS-PAGE and immunoblotting with antibodies to the indicated proteins.

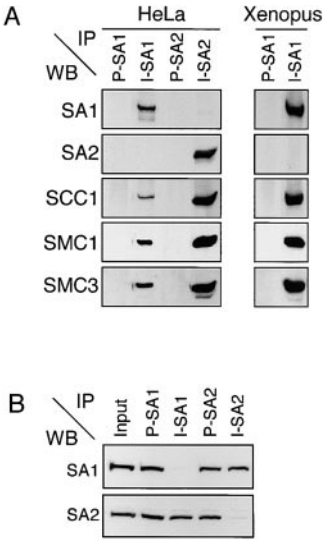

Figure 2. SA1 and SA2 are subunits of two distinct human 14S cohesin complexes. (A) Low-speed supernatant of extracts from logarithmically growing HeLa cells or *Xenopus* interphase egg extracts were analyzed by immunoprecipitation (IP) with either preimmune (P) or immune antibodies (I) against SA1 (444) or SA2 (446) and analyzed by SDS-PAGE and Western blotting (WB) with antibodies to the indicated proteins. (B) HeLa extracts were immunodepleted as in A and the resulting supernatant fractions were analyzed by SDS-PAGE and immunoblotting with SA1 (444) and SA2 (446) antibodies.

analyzed by immunoblotting (Fig. 2 A, left). Both the SA1-specific antibody 444 and the SA2 antibody 446 coprecipitated SCC1, SMC1, and SCC3. However, the SA1 antibodies did not precipitate SA2 and the SA2 antibodies did not precipitate SA1. Likewise, SA1 antibodies depleted SA1 but not SA2, and SA2 antibodies depleted SA2 but not SA1 from cell extracts (Fig. 2 B). These results indicate that SA1 and SA2 assemble with SMC1, SMC3, and SCC1 into two distinct types of 14S cohesin complexes that contain either SA1 or SA2. In density gradient centrifugation experiments, SA1 was also found in fractions that contain 9S cohesin (Fig. 1 B), but coimmunoprecipitation experiments indicated that SA1 is not associated with SMC1 and SMC3 in these fractions (data not shown).

The association of SA1 and SA2 with human cohesin subunits was further confirmed by analyzing the protein composition of SA1/SA2 immunoprecipitates by silver staining. Extracts from logarithmically growing HeLa cells were immunoprecipitated with the antibody 447, which recognizes both SA1 and SA2. After elution of bound proteins with either buffers of low pH (Fig. 3 A) or the antigenic peptide (Fig. 3 C) we observed protein bands corresponding to 160, 140–150, 120, and 85 kD, with the 140–150-kD band often appearing as a doublet (Fig. 3 A). Immunoblot experiments suggested that the 160-kD band contained SMC1, the 140–150-kD doublet SMC3 and SA2 in the lower band and SA1 in the upper band, and the 120-kD band contained SCC1 (Fig. 3 B). None of these proteins could be precipitated with antibodies to other protein complexes such as the APC (Fig. 3 A) or with control immunoglobulins, suggesting that their coprecipitation with SA1 and SA2 is specific.

When immunopurified cohesin complexes were fractionated by sucrose density gradient centrifugation and analyzed by immunoblotting (Fig. 3 B) and silver staining (Fig. 3 C) SA1, SA2, SCC1, SMC1, and SMC3 were found to cofractionate with a sedimentation coefficient of 14S, whereas the 85-kD protein (p85) sedimented less far with a sedimentation coefficient of 13S. Further immunoprecipitation experiments suggested that p85 is not a constitutive subunit of 14S cohesin and that its sedimenatation coefficient of 13S is not due to physical association with cohesin subunits. Instead, this protein may form homo-oligomeric complexes itself (Sumara, I., C. Gieffers, and J.-M. Peters, unpublished results). It therefore remains to be determined if the presence of p85 in SA1/SA2 immunoprecipi-

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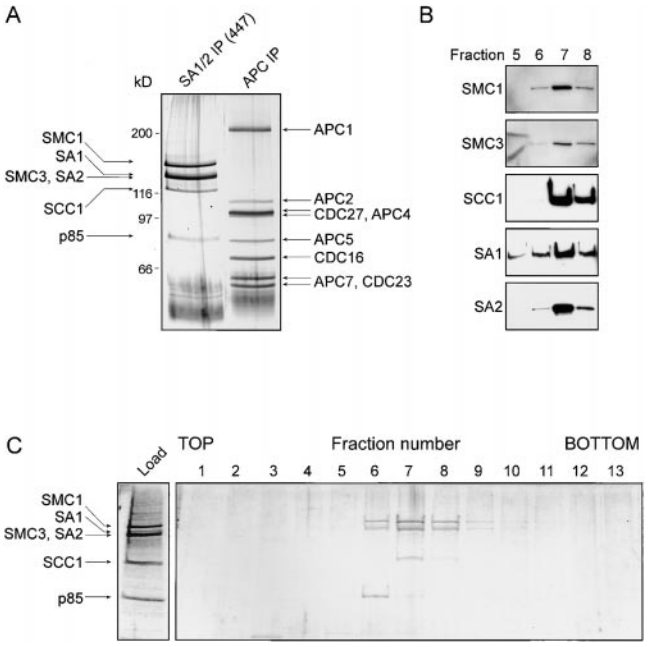

Figure 3. Purification of human cohesin complexes. (A) Immunoprecipitates (IP) obtained with either SA1/2 (447) antibodies or as a control with CDC27 antibodies (APC IP) from low-speed supernatant of extracts from logarithmically growing HeLa cells were analyzed by SDS-PAGE and silver staining. The positions of cohesin subunits, p85 and APC subunits as determined by immunoblotting are indicated. (B) Proteins were eluted with antigenic peptides from cohesin immunoprecipitates obtained as in A and further separated by sucrose density gradient centrifugation. Fractions 5–8 were analyzed by SDS-PAGE and immunoblotting with antibodies to the indicated proteins. (C) Sucrose density gradient fractions from the experiment shown in B were analyzed by SDS-PAGE and silver staining.

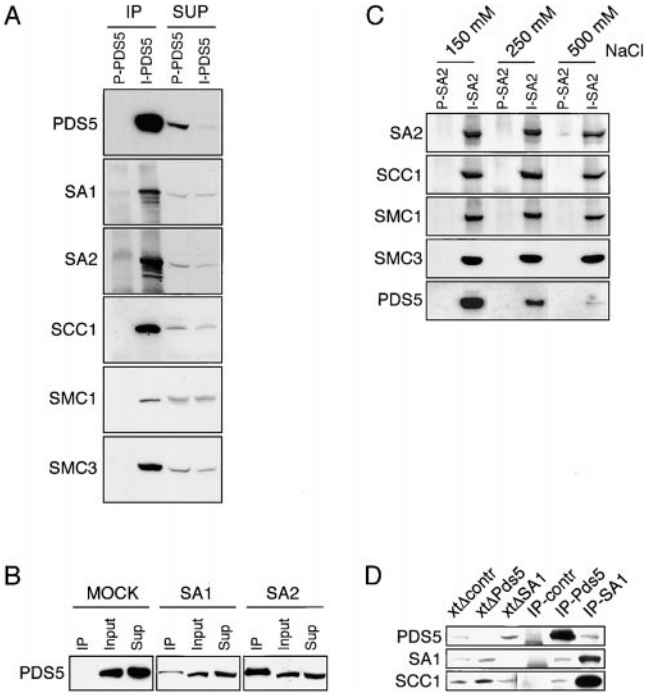

Figure 4. PDS5 is found in association with SA1- and SA2-containing cohesin complexes. (A) Low-speed supernatant of extracts from logarithmically growing HeLa cells was analyzed by immunoprecipitation with either preimmune (P) or immune antibodies (I) to PDS5. Immunoprecipitates (IP) and supernatants (SUP) were analyzed by SDS-PAGE and Western blotting with antibodies to the indicated proteins. (B) HeLa extracts prepared as in A were analyzed by immunoprecipitation with SA1, SA2, or with nonspecific control (MOCK) antibodies and the immunoprecipitates (IP), extracts before immunoprecipitation (Input) and supernatants (Sup) were analyzed by SDS-PAGE and Western blotting with antibodies to PDS5. (C) HeLa extracts prepared as in A were analyzed by immunoprecipitation with either preimmune (P) or immune (I) SA2 antibodies (446). After washing with buffers containing either 150, 250, or 500 mM NaCl, the immunoprecipitates were analyzed by SDS-PAGE and Western blotting with antibodies to the indicated proteins. (D) *Xenopus* interphase extracts were analyzed by immunoprecipitation with either nonspecific (IP-contr), *Xenopus* PDS5 (IP-PDS5), or SA1 (IP-SA1) antibodies. The precipitates were analyzed by immunoblotting with PDS5, SA1, and SCC1 antibodies. After immunoprecipitation with control (xtΔcontr), PDS5 (xtΔPDS5), or SA1 (xtΔSA1) antibodies the resulting supernatants were analyzed side by side. PDS5 antibody 648 was used for the IP and 647 for immunoblotting.

tates reflects a transient association of this protein with 14S cohesin or is due to nonspecific interactions of p85 with the 447 antibodies.

Density gradient centrifugation (Fig. 1 C) and immunoprecipitation experiments (Fig. 2 A, right) using extracts from *Xenopus* eggs showed that *Xenopus* SA1 (see immunoblot in Fig. 1 A) is also associated with SCC1, SMC1, and SMC3. Similar data were obtained when extracts from somatic *Xenopus* cells were analyzed (data not shown). These results suggest that the p155 subunit previously identified in *Xenopus* 14S cohesin (Losada et al., 1998) corresponds to SA1. Because none of our antibodies reacted with a protein similar to SA2 in extracts from *Xenopus* eggs or somatic cells (Fig. 1 A and data not shown) we were unable to test if *Xenopus* eggs also contain an SA2-containing cohesin complex or not.

Human and Xenopus Orthologs of BIMD/Spo76p/Pds5p Are Associated with 14S Cohesin Complexes

In fungi, BIMD/Spo76p/Pds5p has been implicated in chromosome cohesion and condensation. To test if BIMD/Spo76p/Pds5p fulfills these functions as a subunit of 14S cohesin we raised antibodies against KIAA0648, a partial human amino acid sequence identified by random cDNA sequencing (Ishikawa et al., 1998), which is 23% identical and 41% similar to BIMD (Denison et al., 1993). Our antibodies raised against KIAA0648 specifically reacted with the partial KIAA0648 in vitro translation product and recognized a 150-kD polypeptide band in HeLa cell extracts (data not shown). We refer to this protein as PDS5.

When HeLa cell extracts were immunoprecipitated with PDS5 antibodies all known cohesin subunits, including SA1 and SA2, could be detected in the precipitates by immunoblotting, whereas no cohesins could be detected in control precipitates obtained with preimmune immunoglobulins (Fig. 4 A). Conversely, SA1 and SA2 antibodies were able to coprecipitate PDS5 (Fig. 4 B). PDS5 antibodies were not able, however, to deplete cohesin subunits from HeLa extracts, although the majority of PDS5 was removed under these conditions (Fig. 4 A). Likewise, PDS5 could not be immunodepleted with SA1 and SA2 antibodies (Fig. 4 B), suggesting that only small portions of 14S cohesin and PDS5 are bound to each other. Consistent with this possi-

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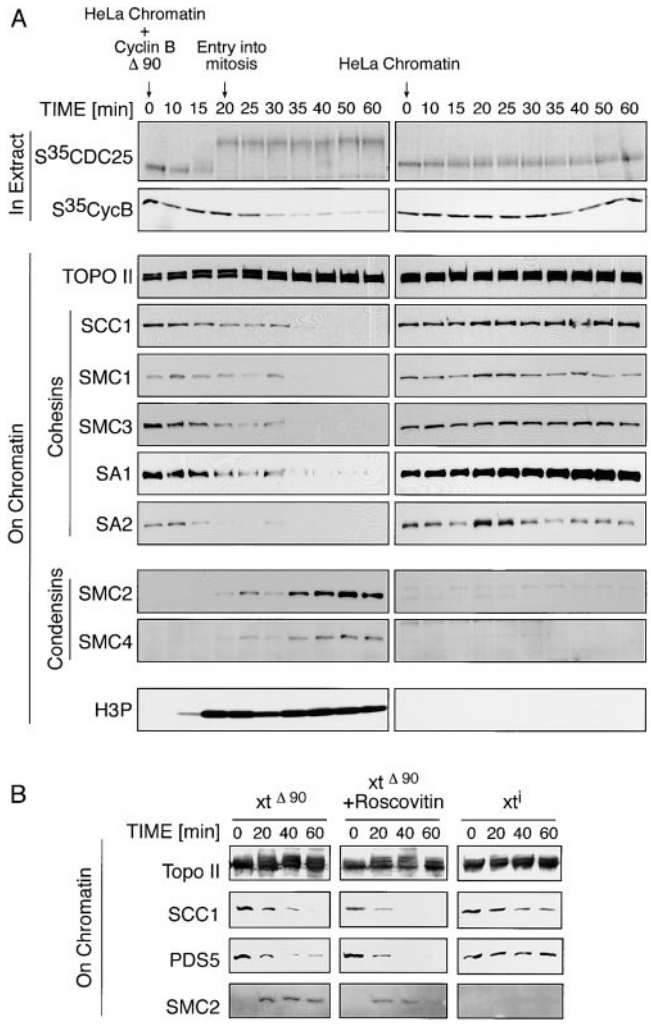

Figure 5. Reconstitution of mitosis-specific dissociation of human 14S cohesin complexes and of PDS5 from chromatin in *Xenopus* egg extracts. (A) HeLa chromatin was incubated in *Xenopus* interphase extract. The extract was either supplemented with nondegradable cyclin B Δ90 to trigger entry into mitosis (left) or left untreated (right). At different time points either extract samples (top) or chromatin reisolated from the extract by sucrose cushion centrifugation (bottom) were analyzed by SDS-PAGE and either PhosphorImaging (top) or immunoblotting with antibodies to the indicated proteins (bottom). The cell cycle state of the extracts was analyzed by monitoring the phosphorylation-dependent electrophoretic mobility shift of ^{35}S-labeled CDC25 and the stability of ^{35}S-labeled cyclin B, which were added to the extracts at time zero. TOPO II, topoisomerase II; H3P, histone H3 phosphorylated on serine 10. (B) HeLa chromatin was incubated in mitotic *Xenopus* egg extract ($xt^{\Delta 90}$), or in mitotic extract treated with 0.8 mM roscovitin ($X^{\Delta 90}$+ Roscovitin), or in interphase extract (xt^{i}). Chromatin bound proteins were isolated at different time points and analyzed as in A.

bility, we found in sucrose gradient centrifugation experiments that only small amounts of PDS5 could be detected in 14S cohesin fractions in long immunoblot exposures (data not shown), whereas the majority of PDS5 sedimented at 9S, i.e., less far than 14S cohesin subunits (Fig. 1 B). PDS5 did not coimmunoprecipitate with SMC1/3 from the 9S fraction (data not shown).

Our finding that only small amounts of PDS5 and 14S cohesin are associated with each other could indicate that PDS5 and 14S cohesin are not stably bound to each other, at least in vitro. Alternatively, the coimmunoprecipitation of PDS5 and 14S cohesin could be due to the presence of residual amounts of chromatin in the HeLa cell lysates to which both cohesins and PDS5 are bound. To test these possibilities we first subjected SA2 immunoprecipitates to different salt conditions and analyzed the behavior of PDS5 and cohesin subunits by immunoblotting (Fig. 4 C). The amount of PDS5 associated with the SA2-containing 14S cohesin complex decreased strongly when the immunoprecipitates were washed with buffers of increased ionic strength and was almost completely abolished in the presence of 500 mM NaCl, whereas the other cohesin subunits remained bound to each other under these conditions (Fig. 4 C). Similar results were obtained for the SA1-cohesin complex (data not shown). PDS5 is therefore easily lost from cohesin immunoprecipitates under stringent washing conditions. In contrast, we found that pretreatment of HeLa extracts with DNAse did not decrease the amounts of PDS5 in cohesin immunoprecipitates (data not shown). We further isolated a partial cDNA for *Xenopus* PDS5 (Vorlaufer, E., and J.-M. Peters, unpublished results) and raised antibodies against this protein. Similar to its human ortholog, *Xenopus* PDS5 sedimented corresponding to 8–9S (Fig. 1 C) but specifically coprecipitated with 14S cohesin (Fig. 4 D). Because *Xenopus* egg extracts contain hardly any chromatin due to the unusually low ratio of DNA to ooplasm in these cells, these results suggest that the association of PDS5 with 14S cohesin is not indirectly caused by independent association of these proteins with chromatin. Our data therefore suggest that PDS5 binds directly and specifically to 14S cohesin complexes but that this association is less stable than the one observed between the cohesin subunits SMC1, SMC3, SCC1, and SA1/2.

SA1- and SA2-containing 14S Cohesin Complexes Dissociate from Chromatin in Prophase and Rebind in Telophase

Yeast cohesin dissociates from chromatin at the onset of anaphase, whereas *Xenopus* 14S cohesin dissociates from chromatin already in prophase. Our observation that human cells contain two distinct 14S cohesin complexes containing either SA1 or SA2 therefore raised the possibility that in vertebrates different cohesin complexes may dissociate from chromatin at different times in mitosis. To test this hypothesis we analyzed the chromatin association of SA1 and SA2 in *Xenopus* egg extracts in vitro (Fig. 5) and in cultured cells by immunofluorescence microscopy in vivo (Fig. 6).

To study the behavior of SA1 and SA2 biochemically, we incubated chromatin from logarithmically growing cultured HeLa cells in *Xenopus* interphase extracts and then stimulated the extracts to enter a mitotic state by adding nondegradable cyclin B Δ90 (Fig. 5). Entry into mitosis was monitored by analyzing the appearance of a mitosis-specific phosphoepitope on histone H3 and by measuring the phosphorylation-dependent electrophoretic mobility shift of the phosphatase CDC25. The chromatin was reisolated at various time points and analyzed for the presence of cohesins and other chromatin proteins by immunoblotting. We used chromatin from human HeLa cells in these experiments because we were unable to detect a *Xenopus* homologue of SA2 with our antibodies (Fig. 1 A) and could therefore not compare the behavior of SA1 and SA2 in a homologous

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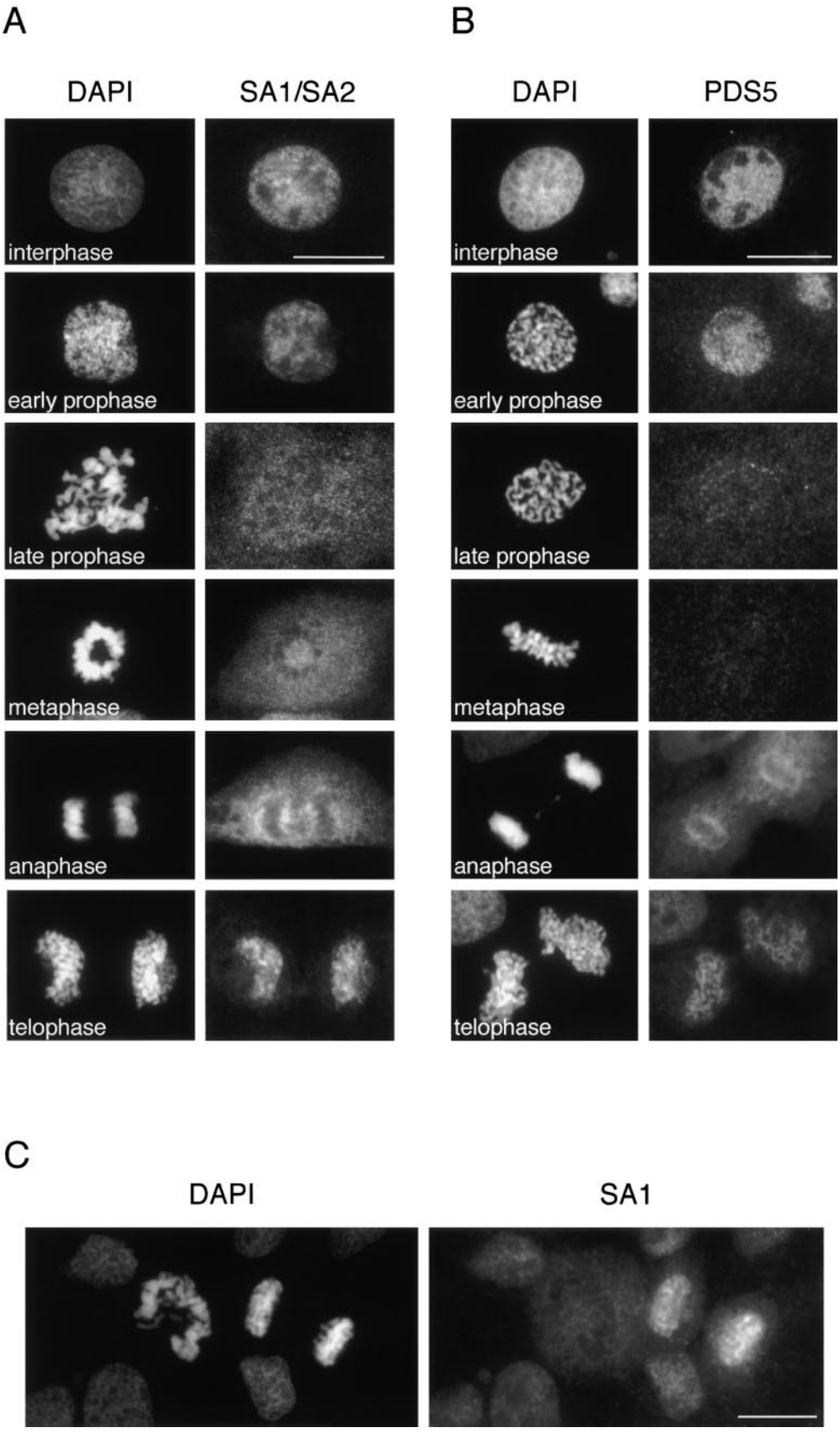

Figure 6. Immunofluorescence microscopy showing the intracellular distribution of SA1, SA2, and PDS5 in human cells at different stages of mitosis. Caco cells were stained with DAPI and with either the SA1/SA2 antibody 447 (A), or with PDS5 antibodies (B), or with the SA1-specific antibody 444. Similar results were obtained with the antibodies 445 and 446 (data not shown). Bars, 5 μm.

Xenopus system. When we compared reactions containing either HeLa chromatin or *Xenopus* sperm nuclei as the chromatin source, both human and *Xenopus* SA1 dissociated with similar kinetics from the chromatin (data not shown), suggesting that physiologically relevant data can be obtained using human chromatin in this assay.

Both SA1 and SA2 dissociated from HeLa chromatin in a mitosis-specific manner (Fig. 5 A and data not shown).

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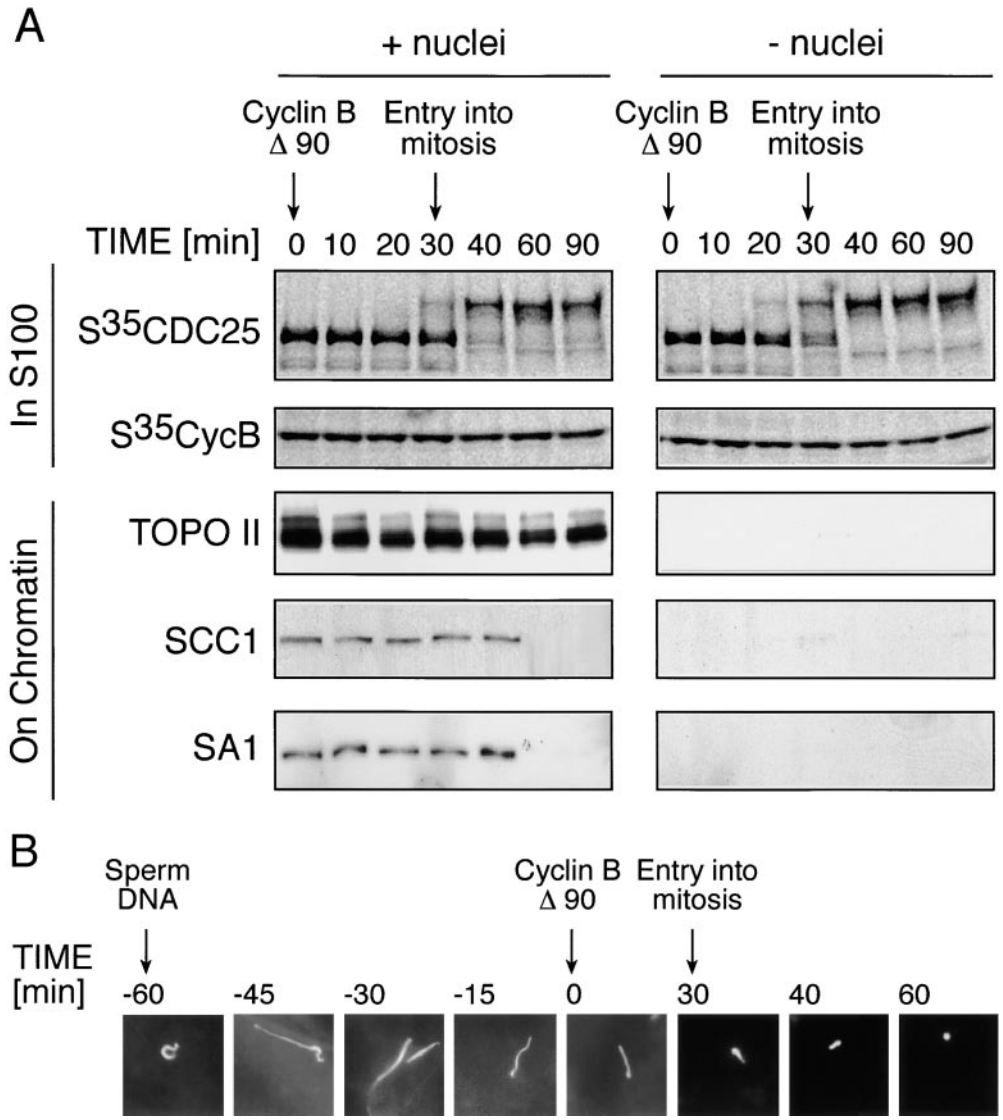

Figure 7. Cyclin B proteolysis is not required for the mitotic dissociation of cohesins from chromatin. (A) High-speed supernatant (S100) fractions of *Xenopus* interphase extracts were incubated for 60 min at room temperature either with 3,200 *Xenopus* sperm nuclei/ul (left) or without nuclei (right). Nondegradable cyclin B Δ90 was then added to trigger entry into mitosis. At different time points either S100 samples (top) or chromatin reisolated from the reaction mixture by sucrose cushion centrifugation (bottom) were analyzed by SDS-PAGE and either PhosphorImaging (top) or immunoblotting with antibodies to the indicated proteins (bottom). The cell cycle state of the extracts was analyzed by monitoring the behavior of ^{35}S-labeled CDC25 and cyclin B as in Fig. 5. TOPO II, topoisomerase II. (B) Immunofluorescence microscopy showing the morphology of sperm nuclei incubated for different time points in S100 fractions as in A and subsequently fixed and stained with DAPI. Note that chromosome condensation still occurs in the S100 fraction.

SA1 and the majority of the cohesin subunits SMC1, SMC3, and SCC1 dissociated from chromatin shortly after the extract had entered a mitotic state. Binding of the condensin subunits SMC2/XCAP-E and SMC4/XCAP-C to chromatin and degradation of cyclin B occurred around the same time (proteolysis was monitored by adding a radiolabeled degradable version of cyclin B as a tracer). SA2 dissociated slightly earlier than the other cohesin subunits, being undetectable on chromatin already at the time when CDC25 and H3 were fully phosphorylated.

The dissociation of SA1 and SA2 from chromatin in mitosis could also be visualized by immunofluorescence microscopy using human cells, but these experiments did not reveal significant differences in the behavior of SA1 and SA2 (Fig. 6, A and C, and data not shown). All SA1 and SA2 antibodies, including the ones that reacted specifically with only SA1 or SA2 in immunoblots, yielded a fine-granular nuclear staining in epithelial human colon carcinoma (Caco) cells in interphase with less staining in nucleolar regions. SA1 and SA2 could also be detected in chromatin regions in early prophase, but no or only little staining was found on chromosomes in late prophase, metaphase, and anaphase, although a halo around separating chromatids was often observed in anaphase. In telophase, both SA1 and SA2 colocalization with chromatin was seen as soon as chromosome decondensation could be observed. Similar data were obtained when cultured human HeLa, mouse EpH4, and rat kangaroo Ptk2 cells were analyzed (data not shown). These results suggest that the bulk of both the SA1- and SA2-containing 14S cohesin complexes dissociates from chromatin in prophase and rebinds in telophase, consistent with earlier observations on SMC1 and SMC3 in *Xenopus* and mouse cells (Losada et al., 1998; Darwiche et al., 1999).

We also analyzed the behavior of PDS5 during the cell cycle by immunofluorescence microscopy (Fig. 6 B). PDS5 was nuclear in Caco cells in interphase, colocalized with condensing chromatin in early prophase but was absent from chromosomes in late prophase, metaphase, and anaphase. As with SA1 and SA2 antibodies, a halo could often be seen surrounding anaphase chromosomes. PDS5 staining reappeared on chromosomes in late telophase. When HeLa chromatin was incubated in *Xenopus* egg extracts, PDS5 dissociated from the chromatin specifically in mitotic extracts (Fig. 5 B). These results suggest that PDS5 is removed from chromatin in prophase and rebinds in te-

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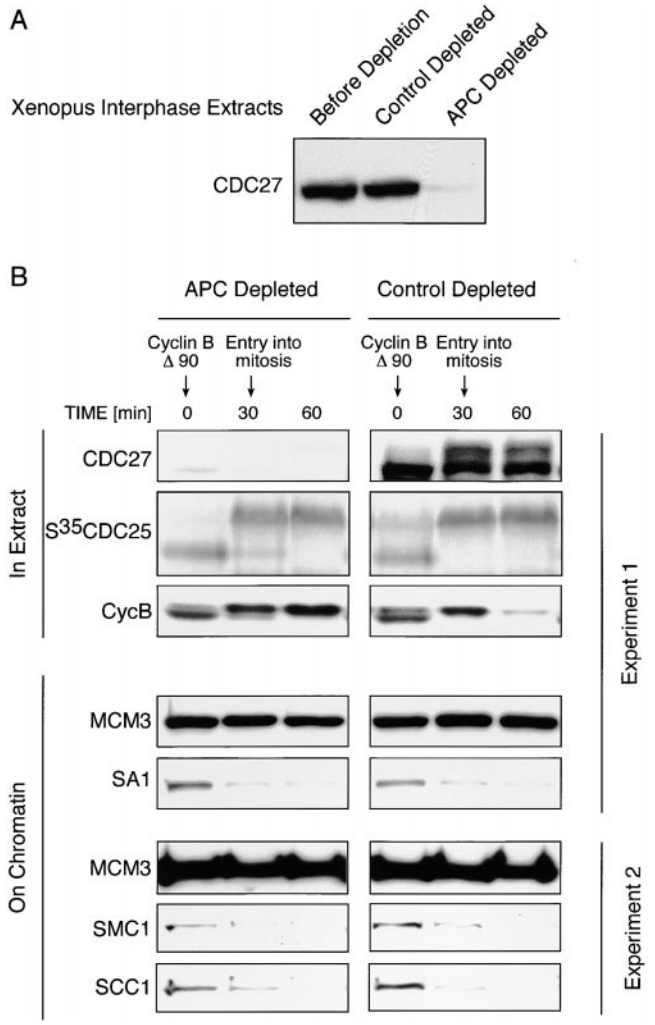

Figure 8. The APC is not required for the mitotic dissociation of cohesins from chromatin. (A) CDC27 immunoblot showing *Xenopus* interphase extract before and after depletion with either control or CDC27 antibodies. (B) APC-depleted (left) and control-depleted (right) *Xenopus* interphase extracts were incubated with sperm nuclei for 30 min and then nondegradable cyclin B Δ90 was added to trigger entry into mitosis. At different time points either extract samples (top) or chromatin reisolated from the reaction mixture by sucrose cushion centrifugation (bottom) were analyzed by SDS-PAGE and either PhosphorImaging (S^{35}CDC25) or immunoblotting with antibodies to the indicated proteins (all other panels). The cell cycle state of the extracts was analyzed by monitoring the behavior of ^{35}S-labeled CDC25 and of endogenous cyclin B. Data from two different experiments are shown. In experiment 2, the degree of APC depletion and the cell cycle behavior of the extracts were the same as in experiment 1 (data not shown).

lophase. PDS5 therefore behaves like 14S cohesin complexes in this respect, further supporting the notion that PDS5 and cohesin subunits interact.

The Dissociation of Vertebrate Cohesin Complexes from Chromatin in Prophase Does Not Depend on the APC

Our biochemical experiments suggested that the dissociation of SA2 from chromatin was initiated before the onset of cyclin B proteolysis, i.e., presumably before the APC is activated, but no clear kinetic difference could be revealed between the dissociation of other cohesin subunits and cyclin B degradation (Fig. 5 A). We therefore tested whether APC activation has a role in cohesin dissociation, as it does in yeast. We first used a partially fractionated extract that is able to enter a mitotic state but unable to activate the cyclin B degradation system. This system uses supernatant fractions obtained by high speed centrifugation of *Xenopus* interphase extract (Félix et al., 1990). Upon addition of cyclin B Δ90 this fraction was able to induce the condensation of *Xenopus* sperm chromatin (Fig. 7 B) and to phosphorylate CDC25 but it failed to degrade cyclin B (Fig. 7 A). Importantly, cohesin subunits dissociated from chromatin under these conditions (Fig. 7 A). Similar observations were made in extracts prepared from *Xenopus* eggs which are arrested in meiosis II by cytostatic factor activity. Although the APC is inhibited in these extracts (Vorlaufer and Peters, 1998) cohesins dissociated from HeLa chromatin incubated in these extracts (data not shown), suggesting that APC activity may not be required for this event.

To further test this hypothesis we immunodepleted the APC from *Xenopus* interphase extracts using antibodies to its subunit CDC27. Immunoblotting experiments indicated that at least 95% of the APC was removed from the extracts by these antibodies (Fig. 8 A). APC could also not be detected in protein extracts from demembranated sperm nuclei that were added as a chromatin source (data not shown), ruling out that the extract was supplemented with APC via this source. Upon mitotic activation, the depleted extracts were still able to phosphorylate CDC25 but could not degrade cyclin B, whereas cyclin B proteolysis occurred in extracts depleted with nonspecific control antibodies (Fig. 8 B). Importantly, the dissociation of cohesin subunits from chromatin occurred normally in the APC-depleted extracts (Fig. 8 B), demonstrating that the APC pathway is not required for this event.

Mitotic CDK1 Activity Is Not Essential for the Dissociation of 14S Cohesin Complexes from Chromatin

Because the dissociation of vertebrate cohesins from chromatin occurs during prophase an obvious candidate for regulating this event is CDK1 whose activation is believed to initiate prophase. We were therefore surprised to see that even high doses of the CDK1 inhibitor roscovitine (up to 0.8 mM) were unable to prevent the mitosis-specific solubilization of cohesins in *Xenopus* egg extracts (Fig. 5 B), although no histone H1 kinase activity could be detected in the extracts under these conditions (data not shown). To further test whether CDK1 activity is required for cohesin dissociation we prepared extracts from cycloheximide-treated *Xenopus* eggs. In these eggs, endogenous B- and A-type cyclins are degraded during exit from meiosis II but new cyclin synthesis is inhibited, resulting in complete inactivation of CDK1. Immunoblot experiments confirmed that neither cyclin A nor cyclin B are detectable in extracts from cycloheximide-treated eggs (Fig. 9 A). Cohesins bound normally to interphase chromatin that was assembled by adding *Xenopus* sperm nuclei to these extracts. By immunoblotting no mitotic cyclins could be detected in

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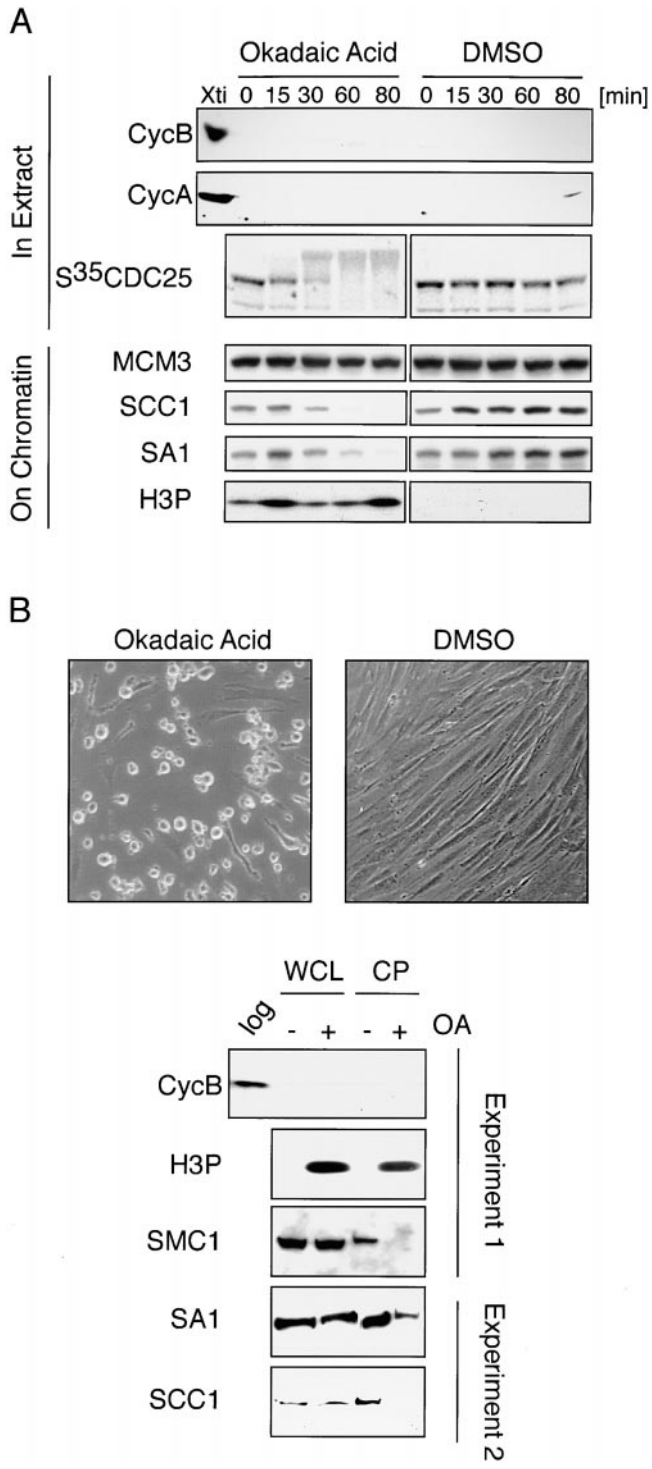

Figure 9. Cohesins can dissociate from chromatin in the absence of CDK1 activity. (A) *Xenopus* sperm nuclei (3,200 nuclei/μl) were incubated for 30 min at room temperature in interphase extracts from cycloheximide-treated *Xenopus* eggs before either 1 μM okadaic acid (left) or DMSO (right) was added. At different time points either extract samples (top) or chromatin reisolated from the reaction mixture by sucrose cushion centrifugation (bottom) were analyzed by SDS-PAGE and either PhosphorImaging (S^{35}CDC25) or immunoblotting with antibodies to the indicated proteins (all other panels). The cell cycle state of the extracts was analyzed by monitoring the behavior of ^{35}S-labeled CDC25 and the phosphorylation of histone H3 on serine 10 (H3P). (B) Phase contrast micrographs of human diploid fibroblasts grown to confluency and then treated for 2.5 h with 10 μg/ml cycloheximide and either with DMSO (right) or with 1 μM okadaic acid (left). Whole cell lysates (WCL) or chromatin pellets (CP) were then analyzed by SDS-PAGE and immunoblotting with antibodies to the indicated proteins (lower panels). Data from two different experiments are shown. OA, okadaic acid; H3P, histone H3 phosphorylated on serine 10.

sperm nuclei, ruling out that they could serve as a source for CDK1 activity. When the phosphatase inhibitor okadaic acid was added, the extract entered a pseudo-mitotic state in which a subset of mitotic events such as CDC25 activation and histone H3 phosphorylation still occurred (Fig. 9 A). Importantly, cohesins still dissociated from chromatin under these conditions, suggesting that CDK1 activity is not essential for this event. The same result was obtained when HeLa chromatin was used as a chromatin source instead of *Xenopus* sperm nuclei (data not shown).

To rule out that these results are specific to the in vitro situation in *Xenopus* extracts, we performed an analogous experiment in human diploid fibroblasts. The fibroblasts were arrested in a quiescent G0-like state by growth to confluency. Under these conditions APC activity is high (Brandeis and Hunt, 1996; Gieffers et al., 1999), resulting in the complete degradation of mitotic cyclins (Fig. 9 B). Subsequently, the cells were treated with cycloheximide to prevent new cyclin synthesis and were stimulated to enter a pseudo-mitotic state by addition of okadaic acid. Under these conditions, the majority of cells rounded up as if entering mitosis and histone H3 was phosphorylated. When chromatin was isolated from these cells, the bulk of cohesins had dissociated, suggesting that cohesins can be removed from chromatin in the absence of CDK1 activity also in human cells in vivo.

Discussion

A substantial body of genetic and biochemical evidence indicates that sister chromatid cohesion depends on a complex of chromosomal proteins, called 14S cohesin. It is less clear if this complex directly connects sisters or enables other proteins to do so, and if the function of 14S cohesin is restricted to sister chromatid cohesion. Despite these uncertainties work in budding yeast suggests that the removal of this complex from chromatin is a prerequisite for anaphase and may in fact be sufficient to allow the separation of sisters. These events depend on activation of the APC–separase pathway which removes cohesin complexes from chromatin by cleaving their Scc1p/Mcd1p subunit (reviewed in Nasmyth et al., 2000). Whether cohesins are regulated similarly in other eukaryotes is not clear because in *Xenopus* and mammalian cells the bulk of cohesin complexes dissociates from chromatin already in prophase, i.e., before the APC–separase pathway is believed to be activated and before sisters separate (Losada et al., 1998; Darwiche et al., 1999; this study). As a first step to address these questions we have purified and characterized cohesin complexes from human cells and *Xenopus* extracts and begun to analyze how their mitotic dissociation from chromatin is regulated.

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Table I. Subunits of 14S Cohesin Complexes and Associated Proteins in Budding Yeast, Xenopus, and Humans

	Yeast	Frog	Human		
14S cohesin core complex	Smc1p	XSMC1	SMC1	SMC1	SMC1
	Smc3p	XSMC3	SMC3	SMC3	SMC3
	Scc1p/Mcd1p	XRAD21	SCC1	SCC1	REC8
	Scc3p	p155	SA1	SA2	SA3/STAG3
Associated proteins	Pds5p	PDS5	PDS5	PDS5	?

The presence of SMC1, SMC3, REC8, and SA3 in a meiosis-specific cohesin complex is hypothetical and has not been experimentally demonstrated yet. In addition to the proteins listed above a 95-kD protein has been observed in immunopurified *Xenopus* cohesin complexes (Losada et al., 1998). The identity of this protein and if it is part of cohesin complexes in other species is unknown.

Human Cells Contain Several Distinct Cohesin Complexes

Our results show that somatic human and presumably many other vertebrate cells contain at least two distinct 14S cohesin complexes. Both of these are composed of the previously identified subunits SMC1, SMC3, SCC1, and in addition either one of two yeast Scc3p homologues, called SA1 and SA2 (Fig. 2, Table I). Our immunoblot data suggest that SA1 is identical with the p155 subunit observed in *Xenopus* 14S cohesin (Losada et al., 1998). After submission of this manuscript, Losada et al. (2000) provided direct evidence that *Xenopus* p155 represents SA1 and also showed that *Xenopus* and human cells contain distinct SA1- and SA2-containing complexes. SA1 and SA2 were previously identified as stromal antigens (Carramolino et al., 1997) but their function was so far unknown. While this work was in progress the identification of a third mammalian Scc3p homologue, called stromal antigen 3 (SA3 or STAG3), was reported (Pezzi et al., 2000). Like the SCC1 homologue REC8 (Parisi et al., 1999), SA3 is expressed exclusively in meiotic cells, suggesting that these proteins may form part of a meiosis-specific cohesion complex (Table I). Unlike SA3, SA1, and SA2 appear to coexist in the same cell types, as both proteins were detected in a number of cultured human, mouse, bovine, and Indian muntjac cell lines and also in nine different mouse tissues tested so far (Sumara, I., B. Peters, and J.-M. Peters, unpublished results). SA1 and SA2 do therefore probably not represent tissue or cell-type specific isoforms. It was therefore attractive to speculate that SA1- and SA2-containing complexes may be regulated differently during mitosis, but we have so far been unable to detect kinetic differences between the solubilization of SA1 and SA2 by immunofluorescence microscopy (Fig. 6). It remains therefore presently unclear why somatic vertebrate cells contain two distinct 14S cohesin complexes and if these complexes perform distinct functions or not. In either case, it is unlikely that these complexes function exclusively in mediating sister chromatid cohesion. Some cohesin subunits have also been identified as part of a mammalian recombination complex (Jessberger et al., 1996), and importantly, vertebrate cohesins rebind to chromatin already in telophase, i.e., before cohesion is established in S phase (Losada et al., 1998; Darwiche et al., 1999; Losada et al., 2000; this study, Fig. 6). Interestingly, SA1 and SA2 are also highly expressed in tissues that are predominantly composed of postmitotic cells such as brain (Sumara, I., and J.-M. Peters, unpublished observation), further suggesting that cohesin complexes may have a rather general role in organizing interphase chromatin, at least in vertebrates.

Our work suggests that 14S cohesin complexes interact with at least one other protein, the previously unidentified KIAA0648 ortholog of *Aspergillus* BIMD, *Sordaria* Spo76p, and budding yeast Pds5p, which we call PDS5. We found that some human PDS5 is bound to both SA1- and SA2-containing 14S cohesin complexes (Fig. 4, A and B), and a similar association between PDS5 and cohesin was seen in *Xenopus* (Fig. 4 D). Like 14S cohesin, PDS5 dissociates from condensing chromatin in prophase and rebinds in telophase (Figs. 5 B and 6 B). This behavior is similar to the one of Spo76p which also leaves chromosomes during prophase (van Heemst et al., 1999). However, unlike other subunits, PDS5 largely dissociates from cohesin complexes in high salt buffers (Fig. 4 C) or during prolonged fractionation procedures (Fig. 1, B and C), suggesting that it may not be part of the 14S cohesin core complex but may rather be weakly bound. Experiments in fungi suggest that BIMD/Spo76p/Pds5p has essential functions in mitotic and meiotic chromosome cohesion, condensation and recombination (Denison et al., 1993; van Heemst et al., 1999; Hartman et al., 2000; Panizza, S., and K. Nasmyth, personal communication). Our results suggest that BIMD/Spo76p/Pds5p performs at least some of these functions by interacting with 14S cohesin complexes. This hypothesis is further supported by the observation that also budding yeast Pds5p is found in association with cohesins (Panizza, S., and K. Nasmyth, personal communication). During the course of this work, a second human homologue of BIMD/Spo76p/Pds5p has been isolated both by random sequencing of large cDNAs (KIAA0979; Nagase et al., 1999) and as an androgen-regulated gene in a prostate cancer cell line (androgene shutoff gene 3/AS3; Geck et al., 1999). We have not yet been able to test if AS3/KIAA0979 also interacts with 14S cohesin complexes because our PDS5 antibodies do not crossreact with this protein, but the high sequence similarity between PDS5/KIAA0648 and AS3/KIAA0979 (62% identity and 75% similarity) makes it likely that such interactions exist.

How and Why Are Cohesins Removed from Condensing Chromosomes in Prophase?

The observation that vertebrate cohesins dissociate from chromatin long before the APC–separase pathway is believed to initiate sister separation (Losada et al., 1998; Darwiche et al., 1999; this study, Fig. 6) was surprising because in budding yeast it is well established that APC activation is essential for the removal of cohesins from chromosomes (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999). One possible interpretation of this result is that in vertebrates the APC becomes active earlier than in

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yeast and that the dissociation of cohesins from chromatin is only one of several steps required for anaphase. This possibility was consistent with the observation that in animal cells the APC^{CDC20} substrate cyclin A is degraded already between pro- and metaphase (Whitfield et al., 1990; Hunt et al., 1992; Edgar et al., 1994; Minshull et al., 1994). Our observation that cohesins can dissociate from chromatin in the absence of cyclin B proteolysis, after depletion of the APC (Figs. 7 and 8) and in meiotic metaphase II extracts where APC is inhibited (data not shown) suggests, however, that the pathway that regulates vertebrate cohesins in prophase is distinct from the APC–separase pathway that controls cohesins in budding yeast. This conclusion is further supported by the observation that the solubilization of cohesin complexes in prophase occurs without detectable SCC1 cleavage (Waizenegger et al., 2000) and that correspondingly the bulk of cohesin complexes is stable throughout mitosis, in contrast to budding yeast Scc1p/Mcd1p, which is completely cleaved and degraded in anaphase (Guacci et al., 1997; Michaelis et al., 1997; Uhlmann et al., 1999; Ciosk et al., 2000).

These results raise several important questions: how and why are vertebrate cohesins removed from chromatin already in prophase, and how are sisters held together between prophase and the onset of anaphase in the apparent absence of cohesins? A possible answer to the latter question is provided by the recent observation that a very small amount of SCC1 remains associated with centromeric regions of human chromosomes until metaphase and that a similarly small amount of SCC1 is cleaved in anaphase, at the same time as SCC1 disappears from centromeres (Waizenegger et al., 2000). Losada et al. (2000) also reported recently that some SA1 staining can be detected between sister chromatids of chromosomes that were assembled in *Xenopus* extracts. These results are consistent with the possibility that in vertebrates residual amounts of cohesins are sufficient to maintain cohesion from prophase to anaphase. These observations further suggest that vertebrate cohesins are regulated by two distinct pathways, a prophase pathway that removes the bulk of cohesin complexes from chromosome arms and a second pathway, activated at the metaphase-anaphase transition, which removes residual cohesin complexes from centromeres. Whereas this model suggests that the APC–separase pathway may be responsible for activating the second pathway, it remains presently unclear how the bulk of cohesins is removed from chromatin in prophase. CDK1 is an obvious candidate for mediating this event. Consistent with this possibility Losada et al. (2000) found that phosphorylation of soluble cohesin complexes by CDK1 decreases their ability to bind to chromatin in vitro. However, CDK1 was not sufficient to induce the dissociation of cohesins from chromatin. Our data further suggest that CDK1 activity is not absolutely essential for the solubilization of cohesins in vivo, at least under conditions where cells are forced to enter a pseudo-mitotic state in the absence of mitotic cyclins (Fig. 9). CDK1 may therefore not be directly responsible for dissociating cohesins, although it may normally well be required to allow progression into the cell cycle state where the prophase cohesin dissociation pathway is activated. Alternatively, it is possible that there is functional redundancy between CDK1 and other mitotic kinases in regulating cohesins in prophase. To clarify these questions will be an important goal for the future.

Likewise, it will be important to understand why cohesins are removed from chromatin already at this early stage of mitosis. An attractive possibility is that cohesins would otherwise topologically interfere with the process of chromosome condensation. This possibility seems plausible since the binding of vertebrate cohesins to unreplicated chromatin in telophase and G1 suggests that these proteins may have a general function in organizing the structure of interphase chromatin. This hypothesis could also explain why the prophase chromatin dissociation pathway has so far not been detected in yeast where only little mitotic chromosome condensation occurs (Guacci et al., 1994). It is furthermore intriguing that the dissociation of cohesins from chromatin in prophase correlates with the appearance of morphologically discernible sister chromatid arms (Sumner, 1991). It is therefore possible that the dissociation of cohesin complexes from chromosome arms in prophase does not only enable condensation but may also begin to release cohesion between chromosome arms.

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