In vitro loading of human cohesin on DNA by the human Scc2-Scc4 loader complex

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The loading of cohesin onto chromatin requires the heterodimeric complex sister chromatid cohesion (Scc)2 and Scc4 (Scc2/4), which is highly conserved in all species. Here, we describe the purification of the human (h)-Scc2/4 and show that it interacts with h-cohesin and the heterodimeric Smc1-Smc3 complex but not with the Smc1 or Smc3 subunit alone. We demonstrate that both h-Scc2/4 and h-cohesin are loaded onto dsDNA containing the prereplication complex (pre-RC) generated in vitro by *Xenopus* high-speed soluble extracts. The addition of geminin, which blocks pre-RC formation, prevents the loading of Scc2/4 and cohesin. *Xenopus* extracts depleted of endogenous Scc2/4 with specific antibodies, although able to form pre-RCs, did not support cohesin loading unless supplemented with purified h-Scc2/4. The results presented here indicate that the *Xenopus* or h-Scc2/4 complex supports the loading of *Xenopus* and/or h-cohesin onto pre-RCs formed by *Xenopus* high-speed extracts. We show that cohesin loaded onto pre-RCs either by h-Scc2/4 and/or the *Xenopus* complex was dissociated from chromatin by low salt extraction, similar to cohesin loaded onto chromatin in G_1 by HeLa cells in vivo. Replication of cohesin-loaded DNA, both in vitro and in vivo, markedly increased the stability of cohesin associated with DNA. Collectively, these in vitro findings partly recapitulate the in vivo pathway by which sister chromatids are linked together, leading to cohesion.

cohesin stability | cell-cycle | cohesion establishment | sister chromatid cohesion

Newly replicated chromosomes are held together until their separation and equal distribution to daughter cells during cell division. Their association is mediated by cohesin, a four-subunit complex comprising (*i*) Rad21/sister chromatid cohesion (Scc)1/multiple chloroplast division (Mcd)1, (*ii*) structural maintenance of chromosomes (Smc)1, (*iii*) Smc3, and (*iv*) Scc3/(stromal antigen) STAG/SA that stably links the sister chromatids together in S and G_2 (1). Smc1 and Smc3 are elongated coiled-coil proteins, each of which folds onto itself to form a globular ATPase head domain through the juxtaposition of the N and C terminus with a hinge domain at the other end. Smc1 and Smc3 interact through their hinge domains to form a heterodimer. The kleisin subunit, Scc1, associates with the head domains of Smc1 and Smc3 to stabilize their interaction and recruits the Scc3/SA subunit. The Smc1-Smc3-Scc1 complex forms a ring structure with an internal diameter of 40 nm, large enough to tether two chromatids (embrace model) (2). Although other models have been proposed to explain how the cohesin ring leads to the association of sister chromatids, substantial evidence supporting the "embrace model" has accumulated (3).

Sister chromatid cohesion is a multistep process that includes cohesin loading onto chromatin and the establishment of cohesion during replication (3). Although cohesin loading required for cohesion occurs before the S phase, the timing of its chromosome association differs among species. In yeast, cohesin binds to chromosomes in G_1, whereas in higher eukaryotes, it is loaded during the telophase stage (3). In *Xenopus* eggs, cohesin is recruited to chromatin after formation of the prereplication complex (pre-RC) (4). Additional factors such as wings-apart like (WapI) and precocious dissociation of sisters-5 (Pds5) are also associated with cohesin on chromatin and prevent cohesin from turning into a "locked" ring before replication. An acetyltransferase [establishment for cohesion (ESCO/Eco)/chromosome transmission fidelity-7 (Ctf7)] catalyzes the acetylation of lysine residues of Smc3 in chromatin-associated cohesin that relieves the antiestablishment activity of WapI/Pds5 (5, 6). It has been proposed that this event occurs as the replication fork passes through the cohesin ring. In higher eukaryotes, Sororin binds to the acetylated cohesin and stabilizes the cohesin–chromatin complex (7). Furthermore, phosphorylation of cohesin by Polo kinase during prophase and cleavage of Scc1 by separase at anaphase are required to release cohesin from chromatin (3).

In addition to its role in sister chromatid pairing, cohesin is also loaded onto DNA at any stage of the cell cycle. Double stranded (ds)DNA breaks induce a damage response that increases the genomic binding of cohesin (8), whereas in yeast, cohesin is recruited to the damaged site (9). The role of cohesin following DNA damage occurs only after acetylation of Scc1 (in yeast) or Smc3 (in vertebrates) (10). Cohesin is also loaded onto chromatin through the action of the transcription apparatus, and its regulatory proteins and studies in *Drosophila*, zebra fish, and humans revealed that it regulates transcription of developmental genes (11). Consistent with these observations, mutations that affect the functions of cohesin have been observed in Cornelia De Lange syndrome (CdLS) and Roberts Syndrome (RBS) causing developmental disorders (12).

The loading of cohesin onto chromatin requires the proteins Scc2 and Scc4 that form a heterodimeric complex. Human (h)-Scc2/Nipped B-like (NIPBL)/minichromosome loss mutants-4 (Mis4) (a 2,804-aa protein, molecular mass of 316 kDa) is highly conserved in all species and contains multiple Huntingtin, Elongation factor 3 (EF3), Protein Phosphatase (PP2A), and the yeast PI3-kinase TOR1 (HEAT) domains involved in protein–protein interactions. Higher eukaryotes possess two forms of Scc2, Scc2A (2,804 aa) and Scc2B (2,697 aa), a splice variant of Scc2A (13). In this report, unless specified, preparations used were a mixture of Scc2A and -B. Scc4 homologs of different species, although highly divergent, share conserved sequences, the tetratricopeptide repeats (TPRs), which are tandemly arranged helices 10–15 aa in length (14). h-Scc4 (651 aa; 66 kDa) has 11 predicted TPRs similar to the *Xenopus* (X)-Scc4. During the cell cycle, Scc2 is found bound to Scc4 as a soluble or chromatin-bound complex. In yeast, analyses of chromatin spreads revealed that cohesin and Scc2/4 were not colocalized (15), suggesting that cohesin dissociates from Scc2/4 after it is loaded onto chromatin and slides to regions distal from its loading site. In yeast, cohesin was shown to translocate from its loading sites to transcriptional termination regions, suggesting that transcription may contribute to its relocation (16).

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Although many studies have described the loading of cohesin onto chromosomes, how cohesin interacts with Scc2/4 and the nature of the chromosomal sites to which they bind are poorly understood. We previously described an in vitro cohesion-loading reaction using *Xenopus* egg extracts (17) and showed that its loading required X-Scc2/4, pre-RCs, and X-Cell Division Cycle (Cdc)7-Dumbbell former (Dbf)-4 related factor (Drf)-1 [Dbf4 Dependent Kinase (DDK)]. We noted that X-Scc2/4 formed a complex with X-DDK and that catalytically active DDK was essential for the loading of X-Scc2/4 and X-cohesin onto pre-RCs. Furthermore, we showed that the recombinant X-Scc2N (1–1,101 aa)/4 derivative was loaded onto pre-RC but unable to support cohesin loading, suggesting that the Scc2 C-terminal region is required for this reaction. We also noted that X-Scc2N (1–1,101 aa) or Scc4 alone did not interact with pre-RCs. In this report, we describe the in vitro loading of purified h-cohesin by h-Scc2/4 onto pre-RCs. We show that h-Scc2/4 purified from HeLa cells interacts with h-cohesin in vitro through the Smc1-Smc3 heterodimer and that purified h-Scc2/4 supports h-cohesin loading onto pre-RCs assembled by X-Scc2-depleted extracts. X-pre-RCs were required for the h-Scc2/4-mediated loading of h-cohesin as geminin, which inhibits pre-RC formation, prevented this reaction. h-Cohesin and h-Scc2/4 loaded onto X-pre-RCs were salt-sensitive, similar to cohesin and Scc2/4 associated with chromatin at the G_1 phase of the cell cycle in HeLa cells. Replication carried out either in vivo or in vitro increased the salt stability of cohesin associated with DNA but did not affect the salt lability of Scc2/4. These findings demonstrate that pre-RCs generated by *Xenopus* extracts can be used for the biochemical characterization of the loading of h-cohesin by the h-Scc2/4 complex.

Results

Isolation of h-Scc2/4 and h-Scc2/4 Derivatives. Using the baculovirus–Sf9 system, we cloned a number of h-Scc2 derivatives containing amino acids 1–500, 1–1,500, and 1–500 plus 1,542–2,375 (N + HEAT), all associated with h-Scc4 (Fig. 1 *A* and *B*). The sedimentation of these complexes through glycerol gradients, followed by SDS/PAGE analysis, revealed that they formed distinct heterodimeric complexes. Previous studies with these truncated Scc2/4 derivatives showed that they bound to pre-RCs but were unable to load cohesin (17). Efforts to isolate full-length Scc2 linked to Scc4 consistently yielded proteolyzed Scc2 fragments linked to Scc4. To overcome this problem, we isolated Scc2/4 from HeLa cells transfected with human N-terminally tagged His_6-$Flag_2$Scc4. The tagged h-Scc2/4 was purified by Flag immunoprecipitation (IP), Heparin chromatography, and glycerol gradient centrifugation. Western blot analysis showed that Scc2 and Scc4 proteins copurified. Silver staining following SDS/PAGE separation of the Heparin column fractions, indicated that the preparation was ~90% pure (Fig. 1*C*). Western blot analysis of glycerol gradient fractions revealed the presence of a peak of Scc2/4 that sedimented between the thyroglobulin (669 kDa) and β-amylase (200 kDa) markers. A portion of Scc2/4 appeared to sediment faster, suggesting the presence of higher molecular mass aggregates (Fig. 1*D*).

Scc2/4 was also purified from HeLa S-100 extracts by ammonium sulfate fractionation, followed by chromatography on mono-Q Sepharose and Heparin columns, followed by glycerol gradient centrifugation. Western blot analysis revealed that the Scc2 and Scc4 proteins copurified and examination of glycerol gradient fractions indicated that h-Scc2 (a mixture of Scc2A and Scc2B) and Scc4 cosedimented (Fig. S1). Coomassie blue staining of SDS/PAGE-separated gradient fractions indicated that the h-Scc2/4 comprised ~50% of the protein present in glycerol gradient

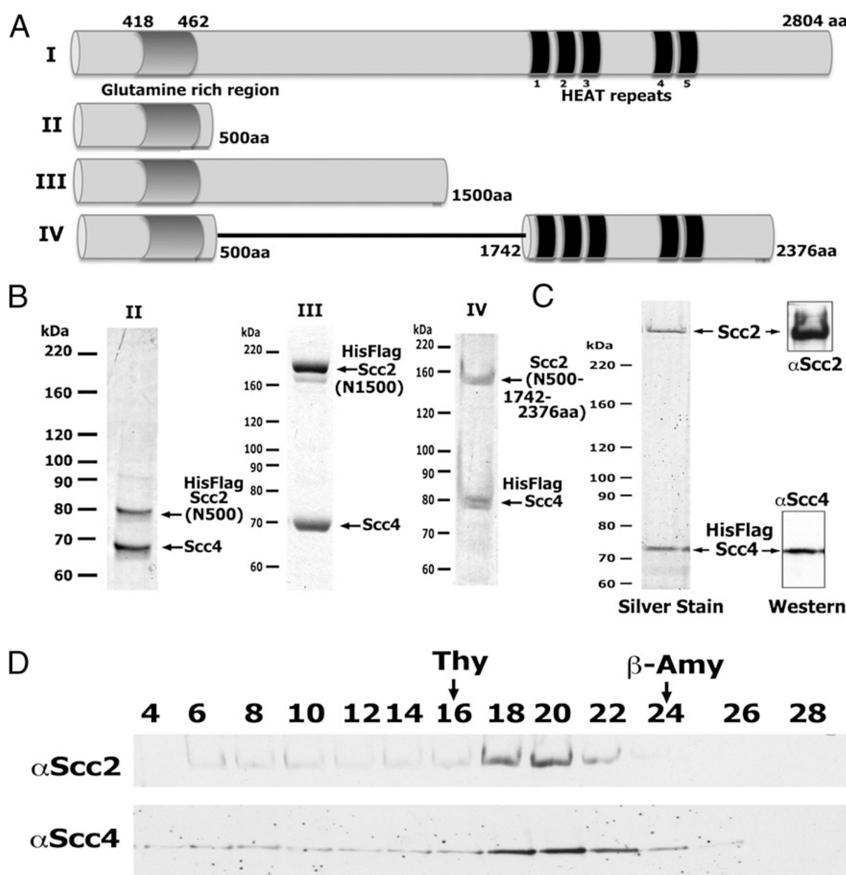

Fig. 1. Isolation of full-length h-Scc2/4 and derivatives. (*A*) Schematic diagram of h-Scc2 showing conserved residues among species and h-Scc2 derivatives expressed and purified in insect cells. (B) h-Scc2 derivatives and h-Scc4 expressed and purified from insect cells. Shown is Coomassie blue-stained SDS/10% polyacrylamide gels containing 0.3 μg of protein from glycerol gradient (GG) peak fractions of His-Flag Scc2 (N500)/Scc4 (GG#28), His-Flag Scc2 (N1500)/Scc4 (GG#22), and Scc2 (N500 + 1,742–2,376 aa/HEAT)/His-Flag Scc4 (GG#24). The roman numerals listed above the lanes correspond to the schematic diagram in *A*. (*C*) HeLa-expressed Scc2/His-Flag Scc4 (0.5 μg) eluted from Heparin column was analyzed by SDS/6.5% PAGE, followed by silver staining (*Left*) and Western blotting with h-αScc2 and h-αScc4 (*Right*). (*D*) Heparin-eluted Scc2/His Flag Scc4 (20 μg) was loaded onto 15–40% (vol/vol) glycerol gradient as described in *SI Materials and Methods*. Shown is the Western blot (to facilitate visualization of the Scc2 and Scc4 bands) of the glycerol gradient fractions.

fraction no. 18 and prominent 160- and ∼60-kDa contaminant proteins were visible. Cohesin (Scc1) and DDK (Cdc7) were detected in glycerol gradient fractions that sedimented as complexes larger than Scc2/4. These findings indicated that the isolated complex contained near stoichiometric levels of h-Scc2 and h-Scc4; DDK and cohesin contaminated the Scc2/4 purified from HeLa cells. Although the purity of the Scc2/4 isolated from transfected HeLa cells was significantly greater than that isolated from S100 extracts, both preparations behaved identically in the experiments described here. Both Scc2/4 preparations were used interchangeably in subsequent experiments.

The hydrodynamic properties of both h-Scc2/4 preparations [using the Monty–Siegel equation (18), which depends on the Stokes radius and sedimentation constant of the complex] indicated that the complex was highly elongated (f/f_o ratio of 2.11) and had an apparent molecular mass 1.45 times greater than that based on its amino acid content. We suggest that the h-Scc2/4 complex is monomeric in structure, similar to that proposed for the yeast Scc2/4 complex (15). Similar analyses of the truncated Scc2/4 complexes indicated they too were monomeric. Collectively, these findings indicate that soluble h-Scc2/4 complexes can be isolated from HeLa cells by standard purification procedures, as well as by transfection of tagged Scc4 into human cells.

h-Scc2/4 Interacts with Cohesin and the Smc1/Smc3 Heterodimer. In addition to h-Scc2/4, we isolated the four-subunit h-cohesin complex (Scc1, Smc3, Smc1, and Scc3/SA1), the stable three-subunit cohesin complex (Smc3, Smc1, Scc1), the two-subunit Smc1-Smc3 heterodimeric complex, and the Smc1 and Smc3 subunits (Fig. 2*A*). We were also able to purify soluble SA1 from baculovirus-infected insect cells. Efforts to isolate other cohesin subcomplex including Scc1, Scc1-Smc1, Scc1-Smc3, or Scc1-SA1 from baculovirus-infected cells were unsuccessful. The Scc1/Rad21 subunit, although expressed well, was insoluble.

In vivo experiments in yeast and humans and in vitro experiments with *Xenopus* extracts demonstrated that Scc2/4 is required for cohesin loading onto chromatin (4, 14, 15, 19). We investigated the in vitro interactions between the four-subunit cohesin and the cloned and purified Scc2/4 derivatives (Fig. 2*B*). Full-length Scc2/4 interacted with cohesin approximately four-times more effectively than the Scc2A (N1500)/4 complex, whereas no interactions were detected with the Scc2 (N500)/4 or Scc2A (N500 + HEAT)/4 derivatives. Incubation of full-length Scc2/4 (containing a mixture of Scc2A and Scc2B) with various cohesin complexes or individual subunits revealed its binding to the three-subunit (Scc1/Smc3/Smc1) and two-subunit (Smc1/Smc3) complexes but not to the Smc1 or Smc3 individual subunit (Fig. 2*C*). Together, these findings indicate that the Scc2 HEAT domains alone (or the HEAT domains associated with the N500 aa) are not sufficient to support the interaction with cohesin and that Scc2/4 interacts specifically with the Smc1-Smc3 heterodimer. We noted that Scc4 complexed with Scc2 derivatives (N500 or the N500 + HEAT domain) did not interact with cohesin, inferring that Scc4 does not bind cohesin.

Loading of Cohesin and Scc2/4 onto *Xenopus* Chromatin Requires the X-Pre-RC. We examined the loading of endogenous X-Scc2/4 onto chromatin by *Xenopus* high-speed soluble (HSS) using a biotinylated 1.4-kb DNA linked to streptavidin magnetic beads as described in *SI Materials and Methods*. Following incubation in the presence or absence of h-Scc2/4 and h-cohesin, the DNA beads were washed and bound proteins were analyzed. In accord with previous results (17), endogenous X-Scc2 and X-cohesin (Smc1) in the *Xenopus* HSS were loaded into chromatin in a pre-RC-dependent manner (inhibited by geminin) (Fig. 3*A*). When reactions were supplemented with purified h-Scc2/4 and h-cohesin, both h- and X-cohesin and h- and X-Scc2/4 were loaded onto chromatin in a pre-RC-dependent reaction (Fig. 3*A*). These findings, however, did not specify which Scc2/4 (X or h) supported cohesin loading. To address this, *Xenopus* HSS extracts were depleted of X-Scc2 (and presumably X-Scc4) with antibodies specific for X-Scc2 [Fig. 3*B*; as previously described (4)]. The X-Scc2-depleted fraction, although capable of forming pre-RC, did not support the loading of endogenous X-cohesin or h-cohesin added to reaction mixtures (Fig. 3*B*). When depleted preparations were supplemented with h-Scc2/4 or h-cohesin alone, only h-Scc2/4 bound to the pre-RC complex. When h-Scc2 and h-cohesin were added together, both complexes were loaded onto the pre-RC (Fig. 3*B*). Thus, pre-RCs formed by *Xenopus* HSS extracts support the chromatin loading of both h-Scc2/Scc4 and h-cohesin, and the loading of the latter required h-Scc2/4.

Cohesin and Scc2/4 Bind to Preformed Pre-RCs. Previous studies revealed that pre-RCs formed by *Xenopus* HSS extracts are stable to high-salt extraction (20) and support in vitro replication following the addition of *Xenopus* nucleoplasmic extracts (NPE), which are unable to synthesize pre-RCs (20–22). We examined whether salt-washed pre-RCs bind Scc2/4 and cohesin (Fig. 4*A*).

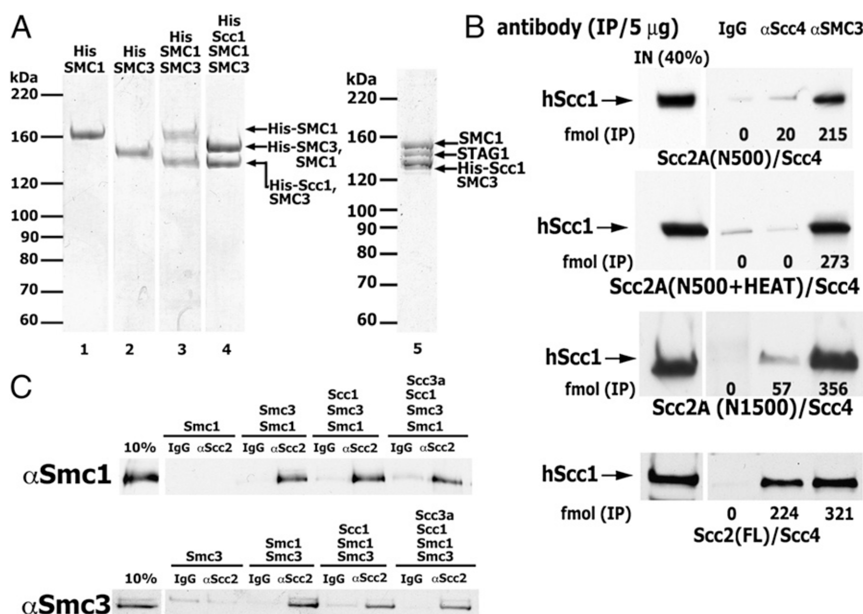

Fig. 2. Interactions between Scc2/4, cohesion, and cohesin derivatives. (*A*) SDS/PAGE analysis of the cohesin complex and various subunits isolated as described in the *SI Materials and Methods*. Lanes 1–3 contained 0.5 μg of the indicated protein, whereas lanes 4 and 5 contained 0.75 μg of protein. Following SDS/10% PAGE separation, gels were stained with Coomassie blue. (*B*) Full-length h-Scc2/4 interacts with h-cohesin. The indicated Scc2/4 derivatives and h-cohesin (1 pmol of each) were mixed and incubated at 25 °C for 30 min, and the mixture immunoprecipitated as described in *SI Materials and Methods*. Protein complexes precipitated with the indicated antibodies (IgG, αScc4, and αSmc3) were separated by SDS/PAGE and then Western blotted with h-αScc1 antibodies. The level of protein pulled down by the antibodies was quantified as indicated. (*C*) Full-length Scc2/4 binds to the Smc1/Smc3 heterodimer. In vitro-transcribed and -translated [^{35}S] methionine-labeled cohesin subunits were mixed with purified full-length Scc2/4 and incubated at 25 °C, followed by IP with antibodies (IgG and αScc2). The Scc2/4-bound material was eluted with SDS loading buffer and separated by SDS/10% PAGE, followed by autoradiography to visualize radiolabeled cohesin subunits.

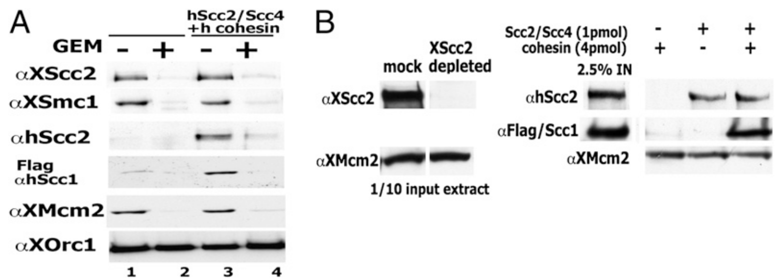

Fig. 3. h-Scc2/4 and h-cohesin are loaded onto X-pre-RCs. (*A*) Reaction mixtures (150 μL), as described in *SI Materials and Methods*, containing *Xenopus* HSS extract, 4 pmol of h-cohesin, 1 pmol of full-length h-Scc2/4, and 400 ng of biotinylated dsDNA bound to magnetic beads in the presence or absence of geminin (400 nM) were incubated for 30 min at 23 °C, and the dsDNA beads were collected and washed as described in the *SI Materials and Methods*. Proteins associated with the biotinylated dsDNA magnetic beads were eluted with 20 μL of SDS loading buffer and applied to SDS/7% PAGE gel and subjected to Western blotting with the indicated antibodies. (*B*) Purified full-length h-Scc2/4 supports loading of h-cohesin onto X-pre-RCs. X-Scc2 was depleted from *Xenopus* HSS extracts using X-Scc2 antibodies as described previously (4). The X-Scc2-depleted HSS extract was supplemented with 1 pmol of full-length h-Scc2/Scc4 and used to assemble the pre-RC in the presence or absence of h-cohesin. Antibodies against h-Scc2, FLAG (for Scc1), and Mcm2 were used to visualize proteins after Western blotting.

Incubation of h-cohesin and h-Scc2/4 with 0.1 M KCl washed pre-RC led to the loading of both human complexes. Furthermore, the loading of h-cohesin onto pre-RCs required h-Scc2/4 (Fig. 4*A*, compare lanes 1 and 3). This reaction was dependent on the presence of pre-RCs because geminin prevented the association of the h-Scc2/4 and h-cohesin (Fig. 4*A*, compare lanes 3 and 4); this suggests that h-Scc2/4 and h-cohesin do not stably bind to duplex DNA. The possibility that other factors required for the loading of the human proteins (particularly X-DDK) onto pre-RCs were associated with the pre-RCs and are stable to the salt extraction, however, cannot be ruled out.

Stability of Human Cohesin and h-Scc2/4 Bound to X-Pre-RC. Studies in higher eukaryotes demonstrated that the stability of cohesin bound to chromatin is altered during the cell cycle. During G_1 to early S phase of the cell cycle, cohesin was shown to associate dynamically with chromatin, whereas during S to G_2, it was converted to a more stably bound form (23). These findings suggest that cohesin becomes "locked" onto chromatin presumably during replication, a change that may require the acetylation of the Smc3 subunit (5). We examined the salt stability of cohesin loaded onto pre-RCs in vitro (Fig. 4*B*) by measuring the association of X-Scc2/4, X-cohesin and X-Mcm2 (pre-RC) following their binding to chromatin and extraction with solutions containing different levels of KCl (50, 75, and 100 mM) (Fig. 4*B*). At 50 mM KCl (the standard condition used in the isolation of complexes), X-Scc2/4, X-cohesin and X-pre-RCs were associated with DNA. Extraction with higher levels of KCl (75 or 100 mM) quantitatively removed both X-Scc2/4 and X-cohesin from the DNA, whereas the pre-RC was substantially retained.

As described in more detail below, the dissociation of cohesin from chromatin at relatively low levels of salt is similar to the in vivo properties of cohesin loaded in G_1 before the onset of replication. We examined the salt extraction properties of X-cohesin following DNA replication. It was recently reported that X-Smc3 subunit of cohesin becomes acetylated after being loaded onto pre-RCs, and this modification did not require replication (24). The acetylated cohesin formed under these conditions was salt labile, but following replication, it was rendered salt-stable. The addition of the CDK inhibitor (p27/Kip), which blocks replication, prevented the salt stabilization of cohesin (Fig. 4*C*) (24). To determine whether DNA synthesis was responsible for this stabilization, experiments were carried out in which pre-RCs were supplemented with nucleoplasmic fraction in the presence of aphidicolin. Addition of aphidicolin, which inhibits deoxynucleotide incorporation but not formation of the preinitiation complex or movement of the replicative helicase (25), prevented the salt stabilization of cohesin (Fig. 4*D*). Thus, DNA synthesis, under the conditions described, contributed importantly to the stabilization of cohesin loaded onto DNA.

We reported previously that active DDK was required for the binding of X-Scc2/4 to pre-RCs. For this reason, we examined whether endogenous X-Cdc7 (reflecting the Cdc7/Drf1 complex in *Xenopus* extracts) also bound to chromatin during formation of pre-RCs. As shown in Fig. S2, X-Cdc7 associated with the DNA was detected. The binding of X-Cdc7, however, did not require the pre-RC formation, as its binding was unaffected by geminin. Surprisingly, the phosphorylated form of Cdc7 was associated with chromatin after extraction of the complex with 100 mM KCl and this stable association was observed only in the presence of pre-RCs. Further studies will be required to determine whether these observations have any bearing on the role that DDK plays in the chromatin loading of Scc2/4 and cohesin.

In Vivo Association of Cohesin with Chromatin Is Cell-Cycle Dependent. We examined whether DNA replication affected the binding of cohesin to chromatin in HeLa cells. Proteins were extracted from the chromatin fraction at different stages of the cell cycle following their release from G_1. As described in the *SI Materials and Methods*, extractions were carried out at different salt concentrations to distinguish cohesin bound weakly to chromatin (salt-sensitive, released by 0.1–0.4 M NaCl extraction) from that bound stably (salt-resistant, released by sonication followed by 1M NaCl extraction). Western blots of salt extracts showed that the level of cohesin (detected by αScc1 and αSmc3) bound stably to chromatin increased as cells progressed from G_1 to S (Fig. 5*A*, compare lanes 3 and 6). Stable association of cohesin with chromatin persisted as cells progressed through G_2 (Fig. 5*A*, lane 9). Interestingly, only chromatin-bound Smc3 was acetylated and was detectable only in S and G_2. We were unable to detect acetylated Smc3 in the soluble fraction (0.1 and 0.4 M NaCl). The association of Scc4 (and presumably Scc2) with chromatin was not stable and did not increase significantly during the cell cycle (Fig. 5*A*, compare lanes 3, 6, 9, and 12). The level of loaded cohesin detected during G_1 was quantified and normalized to the amount of origin recognition complex (ORC) loaded on chromatin (shown in Fig. 5*B*). Approximately 20% of the total cohesin detected was associated stably with chromatin in G_1, and this level increased to 80% as the cells progressed through S and G_2. In mitosis, only 9% of the total cohesin was bound stably to chromatin. These findings show that the fraction of cohesin bound stably to chromatin increased during DNA replication, in keeping with previous reports (23), indicating that the properties of cohesin on DNA changes when cohesion between sister chromatids is established in the S phase.

Discussion

Cohesin is involved in multiple functions including sister chromatid pairing, repair of damaged DNA, and regulation of gene expression; they all require the loading of cohesin onto DNA that depends on the Scc2/4 complex (3).

Previous studies with *Xenopus* proteins revealed that Scc2 and Scc4 interact through their N-terminal regions and the chromatin loading of endogenous Scc2/4 and cohesin in *Xenopus* extracts required the pre-RC and an active DDK. We showed that both truncated and full-length X-Scc2 linked to X-Scc4 interacted with DNA containing pre-RCs, but only full-length X-Scc2/4 supported X-cohesin loading. In this report, we describe the isolation of full-length h-Scc2/4 and truncated h-Scc2 derivatives complexed to Scc4. IP experiments showed that full-length

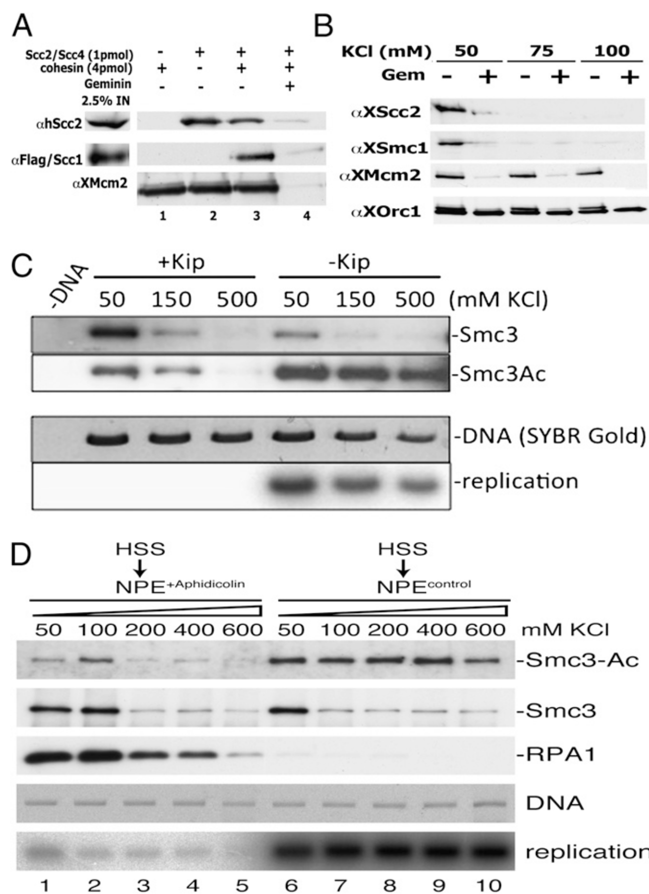

Fig. 4. Salt stability of proteins loaded onto pre-RCs by *Xenopus* HSS extracts. (*A*) Loading of h-cohesin and h-Scc2/4 onto high-salt-washed X-pre-RC on dsDNA magnetic beads pre-RCs that were washed with 100 mM KCl buffer (ELB) were incubated with geminin-treated HSS and 4 pmol of h-cohesin or 1 pmol of h-Scc2/Scc4 for 30 min at 23 °C. The dsDNA magnetic beads were then washed with ELB, and the proteins bound to DNA were analyzed by SDS/PAGE and Western blotting. (*B*) X-pre-RC was assembled on dsDNA magnetic beads as described previously (21). The bead-associated complex was washed with 50, 75, or 100 mM KCl in ELB buffer, and the proteins that remained bound to the beads were analyzed by Western blotting. (*C*) Replication catalyzed by *Xenopus* NPE leads to the stabilization of loaded cohesin. Reactions (40 μL) were incubated with *Xenopus* HSS extracts in the presence of a biotinylated-circular 2.5-kb plasmid at 22 °C or 30 min to assemble pre-RCs and load X-cohesin onto the DNA. Mixtures were then supplemented with NPE and [^{32}P]dATP. Kinase interacting protein (Kip)1 (p27, 0.1 μM) was added to inhibit DNA synthesis, where indicated. DNA synthesis, the determination of level of DNA present in each reaction with SYBR Gold staining, autoradiography, KCl extraction, and immunoblotting were carried out as described previously (24). (*D*) Singly biotinylated 3.0-kb plasmid was immobilized on Sepharose beads and incubated in HSS at 40 ng/μL for 30 min at 22 °C. NPE supplemented with [α-^{32}P]dCTP in the presence of 50 μg/mL aphidicolin (lanes 1–5) or DMSO (control: lanes 6–10) was added into the reaction mixtures and incubated for 2 h. The DNA beads were recovered and washed with buffers containing the indicated KCl concentrations. Bound proteins were eluted in SDS loading buffer and separated in SDS/PAGE, and the protein bands were visualized by Western blotting using the indicated antibodies. DNA was detected by using SYBR Gold, and nucleotide incorporation [α-^{32}P]dCMP was measured by BAS2000 phosphoimager (Fujifilm).

h-Scc2/4 interacted with h-cohesin, whereas the truncated Scc2 derivatives linked to Scc4 bound cohesin either poorly or not at all. We found that full-length h-Scc2/4 interacted with the h-Smc1/Smc3 heterodimer as efficiently as with the four-subunit h-cohesin complex but not with the Smc1 or Smc3 subunit.

The association of cohesin with pre-RCs formed by *Xenopus* extracts required the full-length Scc2/4. Under the conditions used, *Xenopus* extracts that contained endogenous cohesin and Scc2/4 when supplemented with h-cohesin and h-Scc2/4 loaded all four complexes. Although *Xenopus* extracts immunodepleted of Scc2 (and presumably Scc4) generated pre-RCs, they failed to support the loading of endogenous or added h-cohesin. Supplementation of the depleted extract with h-Scc2/4 and h-cohesin resulted in the loading of cohesin onto pre-RCs.

Extraction of pre-RCs with 0.1 M KCl quantitatively removed X-Scc2/4 and X-cohesin but did not dissociate pre-RCs (shown by retention of X-Mcm2). Such salt-washed pre-RCs supported the loading of added h-Scc2/4 and h-cohesin. Collectively, these findings indicate that the binding of cohesin to DNA occurs following its interaction with Scc2/4, which selectively interacts with chromatin-containing pre-RCs. In the absence of Scc2/4, cohesin alone does not bind to pre-RCs. The experiments described here suggest that human and *Xenopus* proteins can be used interchangeably. Thus, X- and/or h-cohesin can be loaded onto X-pre-RCs by h- and/or X-Scc2/4. Quantitative Western blot analyses indicated that the level of cohesin and Scc2/4 loaded onto pre-RCs (each ~10 fmol) was almost equivalent to the level of pre-RC complex added. When the amount of pre-RC added was doubled, the level of cohesin and Scc2/4 loaded increased accordingly, suggesting a stoichiometric rather than catalytic reaction. It should be noted that the interaction of Scc2/4 and cohesin resulted in substantial levels of the complex. In vivo studies indicate that cohesin is extensively loaded at pericentric regions, possibly suggesting that Scc2/4 can act catalytically (15). Whether additional proteins (associated with centromeres or transcription) contribute to the loading of cohesin and influence the activities associated with Scc2/4 remain to be determined. Our efforts to evaluate the role of DDK in the loading reaction revealed that the phosphorylated form of Cdc7 (and possibly Drf1) is associated with pre-RCs. Its role in the loading reaction, however, remains to be further investigated.

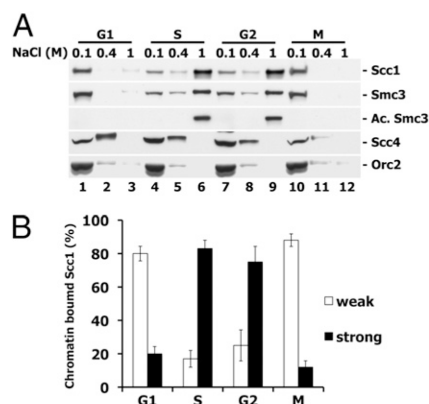

Fig. 5. Stability of cohesin associated with chromatin during the cell cycle. (*A*) Proteins extracted from the chromatin fraction at different salt (NaCl) concentrations and phase of the cell cycle were immunoblotted with antibodies specific for Scc1 (cohesin), Smc3, Ac-Smc3, Scc4 (Scc2/4), or Orc2 (ORC). The preparation of cells and conditions used were as described in the *SI Materials and Methods*. (*B*) The level of chromatin bound cohesin (Scc1) detected in the Western blot shown in *A* was quantified and normalized to the amount of ORC (0.1 M NaCl extract). The percentage of cohesin (Scc1), designated as weak (extracted with 0.1 plus 0.4 M NaCl), and stably associated with chromatin (extracted with 1 M NaCl plus sonication), indicated as strong, are plotted as a function of the cell cycle.

In vivo fluorescence recovery after photobleaching (FRAP) experiments in mammalian cells demonstrated that cohesin binds dynamically to DNA during G_1. Following replication, the residence time of chromatin-bound cohesin was increased (23). In vivo experiments described here with HeLa cells using salt extraction rather than FRAP analysis also revealed a cell cycle-dependent change in the association of cohesin with chromosomes. At G_1, the bulk of the DNA–cohesin complex was dissociated by

0.1–0.4 M NaCl, whereas following replication (G_2), the amount of cohesin associated stably with DNA (1 M NaCl) was increased. Our in vitro experiment showed that cohesin and Scc2/4 loaded on pre-RCs were quantitatively displaced by extraction with 75 or 100 mM KCl, properties similar to Scc2/4 and cohesin bound to DNA in vivo in G_1. Following in vitro replication by the *Xenopus* system (HSS extract plus the nucleoplasmic fraction), cohesin was stably associated with DNA (resistant to 0.6 M KCl). These findings suggest that following replication, stable chromatin-bound cohesin is present in complexes that have established cohesion.

In vivo studies in yeast suggest that Scc2/4 and cohesin colocalized with Pol I, II, and III transcription units along chromosome arms and more abundantly with core centromeric regions (16, 26, 27). Cohesin located at centromeres appeared dependent on specific kinetochore proteins (28). Possibly in accordance with this notion, studies with crude S-phase yeast extracts reported cohesin loading onto specific pericentric DNA regions (29). The loaded cohesin was stably associated with this DNA (0.5 M KCl). Because it is unlikely that such extracts support DNA replication, these findings may be attributable to interactions between cohesin and centromeric proteins. In higher eukaryotes, cohesin and Scc2/4 are found associated with CCCTC-binding factor (CTCF) transcription complex (30) and with aborted transcription complexes at or near promoters in *Drosophila* (11). Together, these observations suggest that Scc2/4 interacts with various protein–DNA complexes at which cohesin and its loading complex are deposited. The specific requirements governing Scc2/4 binding, however, remain to be defined.

It has been proposed that Scc2/4 facilitates the loading of cohesin onto DNA by stimulating the hydrolysis of ATP bound to the head domains of Smc1 and Smc3 (26). This step is then followed by the passage of DNA strands within the cohesin ring and ring closure (DNA entrapment). In vivo studies in yeast with cohesin mutants containing Smc1 or Smc3 subunits unable to hydrolyze ATP (Smc1, E1158Q; Smc3, E1155Q) showed that Scc2/4 loaded these mutant cohesins onto DNA. However, they remained colocalized and failed to support cohesion, in contrast to wild-type cohesin (26). Our results indicate that before replication, Scc2/4 and cohesin are bound weakly to chromatin, and it is unclear whether cohesin is linked to DNA directly or indirectly (via Scc2/4). Following replication, cohesin was found to be stably associated with DNA, whereas Scc2/4 was not. These changes suggest that cohesin has participated in a DNA entrapment reaction. Several lines of evidence indicate that the mini-chromosome maintenance (Mcm) complex (loaded as the pre-RC) encircles dsDNA (31). It is likely that this architecture contributes to the stability of the Mcm complex following washings with high-salt buffers. Although the salt stability of an entrapped cohesin–dsDNA complex is unknown, we conjecture that it is likely to be the same as an Mcm-encircled duplex DNA. If this were the case, it would suggest that other factors, in addition to Scc2/4, are required to stabilize the cohesin–chromatin complex. The availability of a soluble Scc2/4 complex should permit in vitro experiments to further define the loading of cohesin onto chromatin.

Materials and Methods

The experimental details are summarized in the *SI Materials and Methods*. This includes cDNA cloning, protein expression and purification, protein interaction assays, X-pre-RC assembly, h-Scc2 and h-cohesin loading, and HeLa experiments. Additional experimental information is included in the figure legends.

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