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Analysis of the role of Aurora B on the chromosomal targeting of condensin I

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ABSTRACT

During mitosis, chromosome condensation takes place, which entails the conversion of interphase chromatin into compacted mitotic chromosomes. Condensin I is a five-subunit protein complex that plays a central role in this process. Condensin I is targeted to chromosomes in a mitosis-specific manner, which is regulated by phosphorylation by mitotic kinases. Phosphorylation of histone H3 at serine 10 (Ser10) occurs during mitosis and its physiological role is a longstanding question. We examined the function of Aurora B, a kinase that phosphorylates Ser10, in the chromosomal binding of condensin I and mitotic chromosome condensation, using an *in vitro* system derived from *Xenopus* egg extract. Aurora B depletion from a mitotic egg extract resulted in the loss of H3 phosphorylation, accompanied with a 50% reduction of chromosomal targeting of condensin I. Alternatively, a portion of condensin I was bound to sperm chromatin, and chromosome-like structures were assembled when okadaic acid (OA) was supplemented in an interphase extract that lacks Cdc2 activity. However, chromosomal targeting of condensin I was abolished when Aurora B was depleted from the OA-treated interphase extract. From these results, it is suggested that Aurora B-dependent and Cdc2-independent pathways of the chromosomal targeting of condensin I are present.

INTRODUCTION

At the onset of mitosis, dispersed chromatin in the interphase nucleus is resolved and packaged into physically separate compact structures, called mitotic chromosomes. This step, mitotic chromosome condensation, is a prerequisite for the accurate segregation of chromosomes, the failure of which is considered to lead to aneuploidy, cancer and cell death. Despite its importance, the molecular mechanism underlying dynamic changes of the higher order chromosome structure is poorly understood for a long time (1,2).

However, considerable progress has been made in the identification of proteins implicated in this process, using yeast genetics or an *in vitro* system derived from *Xenopus* egg extract. For example, topoisomerase II and condensin(s) have been identified as essential factors required for mitotic chromosome condensation. Topoisomerase II is an enzyme that catalyzes the strand passage of double-stranded DNA (3), and its activity is required for the proper condensation of mitotic chromosomes, as demonstrated by yeast genetics and the *Xenopus in vitro* system. Condensin(s) plays a more direct role in mitotic chromosome condensation, and also in the maintenance of its structure. About a decade ago, 13S condensin (now referred to as condensin I) was identified using a *Xenopus* system (4,5). Condensin I is composed of two structural maintenance of chromosomes (SMC), SMC4/CAP-C and SMC2/CAP-E subunits and three other non-SMC (CAP-D2, -G and -H) subunits, all of which are conserved widely in eukaryotes, ranging from yeast to humans (6–9). Condensin I, purified from either *Xenopus* mitotic egg

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extract or mitotic HeLa cells, possesses activity to induce positive superhelical tension in double-stranded DNA in an ATP-hydrolysis-dependent manner (10–12). This activity is stimulated by mitosis-specific phosphorylation and reduced by CK2-mediated interphase phosphorylation, which suggests that it may be a physiologically relevant activity required for mitotic chromosome condensation (13,14). More recently, a second condensin complex, condensin II, which possesses the same set of SMC subunits as condensin I but contains a different set of non-SMC subunits (CAP-D3, -G2 and -H2), was identified (15,16). The two complexes show distinct distributions on the mitotic chromosome axis and have distinct functions during mitotic chromosome condensation (15,17,18).

All eukaryotic chromosomes condensed during mitosis, and two mitotic kinases are implicated in the establishment and maintenance of chromosomes in a condensed state by regulating condensin complexes. One pathway is mediated by the master mitotic kinase, Cdc2. In *Xenopus* or humans, biochemical activity and the chromosomal association of condensin are stimulated by Cdc2 (4,13). In fission yeast, the phosphorylation of SMC4 by Cdc2 is required for nuclear localization of the condensin holo complex (19). The other pathway is thought to require Aurora B kinase (Ipl1 in budding yeast); however, the role of Aurora B in the regulation of condensin has been less characterized. Aurora kinases are serine/threonine protein kinases that play critical roles in many aspects of cell division, including the centrosome cycle, spindle assembly, chromosome condensation, the spindle checkpoint and cytokinesis (20,21). In mammals, at least three Auroras (A, B and C) are present and they share similar structures, but differ in their expression patterns, subcellular localization and substrates. Among them, Aurora B phosphorylates histone H3 at its serine 10 (Ser10) residue during mitosis, which may be related to mitotic chromosome condensation. Aurora B forms a tight complex with inner centromere protein (INCENP) and survivin, and more recently, a fourth subunit of Aurora B complex was identified (22,23). It is also reported that Aurora C phosphorylates Ser 10 of nucleosomal H3 (24).

Formerly, Aurora B was believed to be implicated in the chromosomal localization of condensin I and mitotic chromosome condensation. Inhibition of Aurora B function results in the loss of H3 phosphorylation and the recruitment of condensin I to chromatin in *S. pombe* (25,26). Depletion of Aurora B by RNA interference (RNAi) blocks mitotic chromosome condensation and the targeting of condensin in *Drosophila* (27) and *C. elegans* (28,29); however, this notion is now challenged. For example, condensin I was normally targeted to mitotic chromosomes in the Aurora B-depleted *Xenopus* mitotic egg extract, in which Ser10 phosphorylation was almost abolished (30,31). Thus, different results were obtained among different species and different experimental systems, and the exact role of Aurora B in mitotic chromosome structures remains to be determined.

In this study, we focused on the regulation of the more abundant form of condensin, condensin I, by Aurora B, using cell-free extracts derived from *Xenopus* eggs. Chromosomal targeting of condensin I was decreased to about 50% by the depletion of Aurora B from mitotic *Xenopus* egg extract. A substantial amount of condensin I was targeted to sperm chromatin in the okadaic acid (OA)-treated interphase extract that lacks Cdc2 activity, accompanied by H3 phosphorylation. Furthermore, partial chromosome condensation was introduced into the OA-treated interphase extract. The OA-dependent chromosomal targeting of condensin I, and H3 phosphorylation were almost abolished by the depletion of Aurora B from the extracts. Taken together, we conclude that a subpopulation of condensin I is bound to chromosomes in an Aurora B-dependent manner.

MATERIALS AND METHODS

Antibodies

Rabbit antisera were raised against synthetic peptides corresponding to the carboxy-terminal sequences of *Xenopus* Aurora A (KNSQLKKKDEPLPGAQ), Aurora B (SRRVLPPVYQSTQSK), INCENP (SNRHHLA VGYGLKY) and Cdc2 (KSSLPDNQIRN). An amino-terminal cysteine was added to each peptide for sulfhydryl coupling. Conjugation of the peptides to keyhole limpet hemocyanin and affinity purification of antibodies was performed as described (32). Affinity-purified antibodies were diluted to a concentration of 1 μg/ml, and used for immunoblotting. Two micrograms of each antibody were used for immunoprecipitation from 100 μl of *Xenopus* egg extracts, as described previously (4).

An antibody that recognizes the phosphorylated form of histone H3 at Ser10 (purchased from Cell Signaling, Danvers, MA, USA) was diluted 1:1000 and used for immunoblotting.

Preparation of *Xenopus* egg extracts

Mitotic and interphase high-speed supernatants were prepared as described previously (4), and was used throughout the study. In some experiments, OA was supplemented into the interphase extract.

Immunodepletion

For immunodepletion of Aurora A from *Xenopus* egg extracts, 10 μg of affinity-purified anti-Aurora A antibody was incubated with 30 μl of Protein A-Sepharose beads for 1 h. To deplete Aurora B, a mixture containing 10 μg of anti-Aurora B and 10 μg of anti-INCENP was used. For Cdc2 depletion, 10 μg of anti-Cdc2 was used. After washing the antibody-coupled beads with XBE2 [10 mM K-Hepes (pH 7.7), 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM K-EGTA (pH 7.7) and 1.7% sucrose], 100 μl of *Xenopus* egg extracts supplemented with the ATP-regenerating system (1 mM Mg-ATP, 10 mM creatine phosphate and 50 μg/ml creatine kinase) were mixed, and incubated on a rotating wheel at 4°C for 1 h. The supernatants were recovered and then incubated with a fresh batch of the same beads. After incubating for 1 h, the supernatant was recovered and used as a depleted extract.

Biochemical analysis of chromatin or chromosomes assembled *in vitro*

Sperm nuclei (5000 nuclei/μl) were incubated with high-speed supernatant supplemented with the ATP-regenerating system at 22°C for 2 h. Samples were placed on ice for 10 min, and chromatin was isolated by centrifugation through a 30% sucrose cushion in XBE2 at 10,000 × *g* for 15 min. The chromatin fraction was subjected to SDS-PAGE followed by Coomassie staining or immunoblotting. In some experiments, the amounts of chromosome-bound condensin subunits in each extract were quantitated using the LAS 1000 imaging system (Fujifilm, Allendale, NJ, USA).

Immunofluorescence staining

Immunofluorescence staining of chromatin or chromosomes assembled in the extracts was performed as described with minor modifications (33,34). For fixation, 1% paraformaldehyde was used rather than 2%. For the detection of condensin I, 1 μg/ml affinity-purified anti-XCAP-H antibody was used.

Purification of *Xenopus* condensin I

Xenopus condensin I was purified from mitotic, interphase or 4 μM OA-supplemented interphase egg extracts using the anti-XCAP-G antibody coupled to protein A-Sepharose beads, as described previously (10).

RESULTS

Depletion of Aurora B from mitotic extract results in the reduction of chromosomal association of condensin I

Aurora is a family of serine/threonine protein kinases that regulates many aspects during cell division. Among them, Aurora B, which phosphorylates nucleosomal Ser10 during mitosis, has long been believed to be correlated with mitotic chromosome condensation and segregation; however, different results are obtained depending on the experimental systems. To re-evaluate the exact roles of Aurora B in chromosome condensation and chromosomal targeting of condensin complex, we used an *in vitro* system derived from *Xenopus* egg extract. There are two types of the *Xenopus* egg extracts; one is a high-speed supernatant in which chromosomes are assembled without preceding DNA replication, the other is a low-speed supernatant that allows one complete round of DNA replication before entry into mitosis. Chromatin assembly using the latter extracts may reflect more physiological process (35); however, we used a high-speed supernatant throughout this study because it is more simple system for analyzing the chromosomal loading of condensin I.

We immunodepleted Aurora B, Aurora A and Cdc2 from mitotic egg extracts, and examined Ser10 phosphorylation, chromosomal targeting of condensin I, and chromosome structures (Figure 1). The immunodepletion efficiency of each kinase was estimated to be more than 94% by quantitative immunoblotting (Figure 1A). First, the phosphorylation level of histone H3 at Ser10 residue was examined. Aurora A depletion barely affected the phosphorylation of Ser10 (Figure 1B, lanes 11–13). When Aurora B was depleted from mitotic extract, the phosphorylation level of Ser10 of the chromatin-bound H3 was greatly reduced to that in the interphase extract (Figure 1B, lower, lanes 8–10), while Aurora B depletion had little impact on the phosphorylation of Ser10 of H3 in the extracts (Figure 1B, upper, lanes 8–10). These results demonstrate that Aurora B is the major kinase phosphorylating Ser10 of nucleosomal H3, while other kinase(s) are also able to phosphorylate the soluble form of H3 at Ser10 in *Xenopus* egg extract. Cdc2 depletion resulted in a loss of Ser10 phosphorylation of chromatin-bound and soluble forms of H3 (Figure 1B, lanes 14–16), suggesting that Ser10 phosphorylation during mitosis is downstream of Cdc2. Next, we tested the chromosomal targeting of condensin I (Figure 1C). Sperm chromatin was incubated with each extract, isolated through a sucrose cushion, and the chromatin-associated condensin I was analyzed by immunoblotting using XCAP-E and XCAP-G. Condensin I was bound to sperm chromatin in a mitosis-specific manner, and the association was almost abolished by Cdc2 depletion (Figure 1C, lanes 14–16). Aurora B depletion resulted in an approx. 50% reduction of chromosomal targeting of condensin I subunits (Figure 1C, lanes 8–10). On the other hand, the chromosomal targeting of condensin I was barely affected by mock depletion or Aurora-A depletion (Figure 1C, lanes, 5–7 and 11–13). Alternatively, chromatin-associated proteins were analyzed by Coomassie blue staining (Supplementary Figure 1A), and the intensity of the bands of XCAP-C, -E and -G was quantitated using the Las 1000 imaging system (Fujifilm) (Supplementary Figure 1B). This experiment also indicates that Aurora B depletion resulted in an about 50% reduction of chromosomal targeting of condensin subunits (Supplementary Figure 1B, lanes 1, 7 and 11). However, it is questionable that the 50% reduction of chromosomal association of condensin I in the Aurora B-depleted extract is a direct effect of the loss of Aurora B. For example, it is possible that Aurora B-depletion results in the reduction of Cdc2 activity in the Aurora B-depleted mitotic extract, which may lead to decreasing the amount of chromosome-associated condensin I. To address the criticism, we compared the Cdc2 kinase activity between control and Aurora B depleted-mitotic egg extract by MPM2 blotting. But, no significant difference was observed (data not shown).

The assembled structures were then fixed and stained with Hoechst to test the effect of the depletion of each kinase on chromosome condensation (Figure 1D). Sperm chromatin was converted into a condensed chromosome-like structure in a mitotic extract (Figure 1Db), but no condensation occurred in the interphase (Figure 1Da) or Cdc2-depleted mitotic extract (Figure 1Df). When Aurora B was depleted from mitotic extracts (Figure 1Dd), the chromosome structure was almost indistinguishable from undepleted (Figure 1Db) or mock-depleted mitotic extracts (Figure 1Dc). These results indicate that chromosome condensation is dependent on Cdc2, but occurs in the Aurora B-depleted mitotic extract, in which the

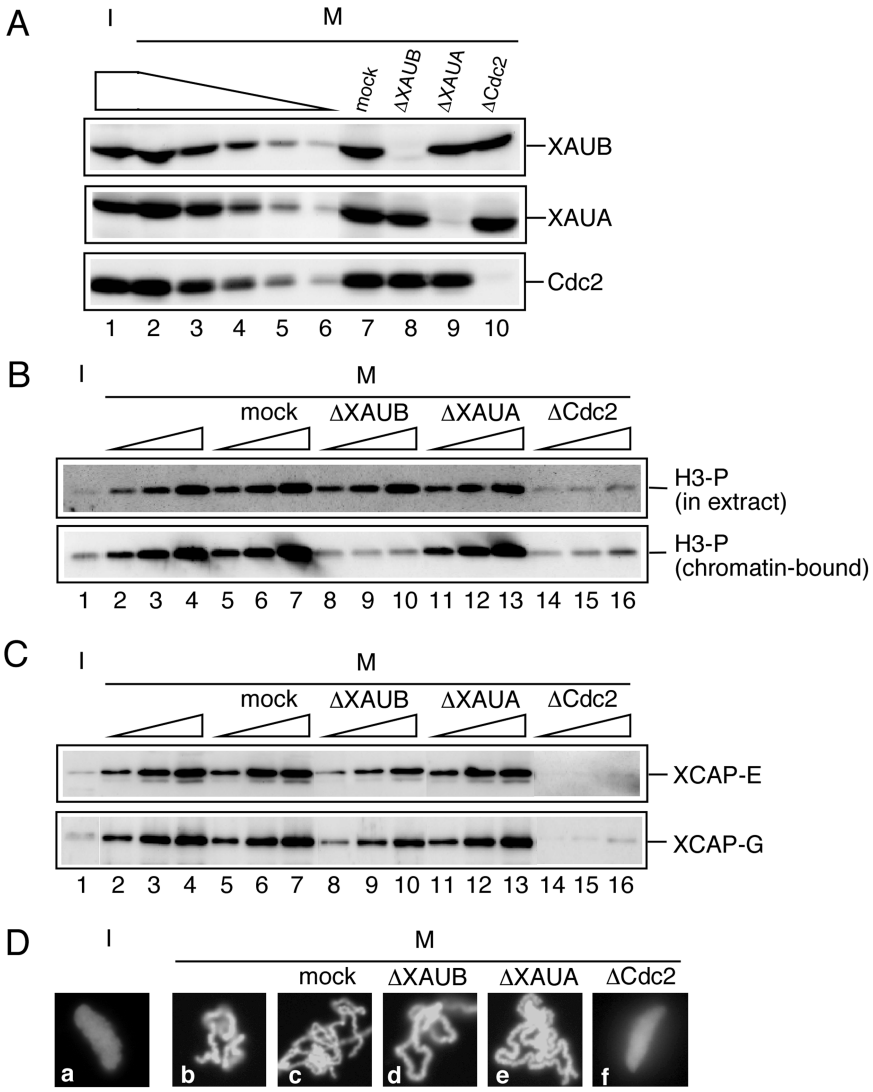

Figure 1. Effect of Aurora-B depletion on condensin I binding to chromosomes. (**A**) Mitotic extracts were depleted with control IgG (lane 7), anti-Aurora B (lane 8), anti-Aurora A (lane 9) and anti-Cdc2 (lane 10). Two microliter aliquots of each extract were analyzed by immunoblotting with the indicated antibodies. To estimate the efficiency of depletion, 2 µl (lane 1) of interphase extract, 2 µl (lane 2), 1 µl (lane 3), 0.5 µl (lane 4), 0.25 µl (lane 5) and 0.12 µl (lane 6) of mitotic extract were loaded in parallel (lanes 7–10). **(B)** Sperm nuclei were incubated with interphase extract (lane 1), mitotic extract (lanes 2–4), mock-depleted mitotic extract (lanes 5–7), Aurora B-depleted mitotic extract (lanes 8–10), Aurora A-depleted mitotic extract (lanes 11–13), or Cdc2-depleted mitotic extract (lanes 14–16). Assembled chromatin and chromosomes were isolated through a sucrose cushion, and 12.5% (lanes 2, 5, 8, 11 and 14), 25% (lanes 3, 6, 9, 12 and 15) and 50% (lanes 1, 4, 7, 10, 13 and 16) were separated by SDS-PAGE, and then detected by immunoblotting using anti-phospho H3 antibody (chromatin-bound; lower). To test the phosphorylation level in the extracts, 1 µl (lanes 2, 5, 8, 11 and 14), 2 µl (lanes 3, 6, 9, 12 and 15) and 4 µl (lanes 1, 4, 7, 10, 13 and 16) of the extracts were immunoblotted with anti-phospho H3 antibody (in extract; upper). **(C)** Chromatin-bound proteins were prepared the same as (**B**), and were separated by SDS-PAGE and immunoblotted by anti-XCAP-E (upper) or anti-XCAP-G antibodies (lower). **(D)** Sperm chromatin was incubated in interphase (a), mitotic (b), mock-depleted mitotic (c), Aurora B-depleted mitotic (d), Aurora A-depleted mitotic (e) or Cdc2-depleted mitotic (f) extracts at 22°C for 2 h, fixed, and then stained with Hoechst.

chromosomal association of condensin I is reduced and Ser10 phosphorylation is abolished.

OA treatment of interphase extract results in the partial chromosome association of condensin I

Figure 1 has shown that some populations of chromosomal targeting of condensin I were regulated by Aurora B; therefore, we next tested whether condensin I is able to target to chromosomes in the absence of Cdc2, when downstream kinases, such as Aurora B, are activated. It is known that the addition of OA, an inhibitor of type 1 and type 2A phosphatases, into interphase extracts promotes the activation of downstream mitotic kinases in the absence of Cdc2 activity. We therefore supplemented OA into the interphase egg extract and examined its effects (Figure 2). The phosphorylation of chromatin-associated H3 at Ser10 in the OA-treated interphase extract reached a similar level to that in the mitotic extract (Figure 2A). This result is consistent with the previous report, in which

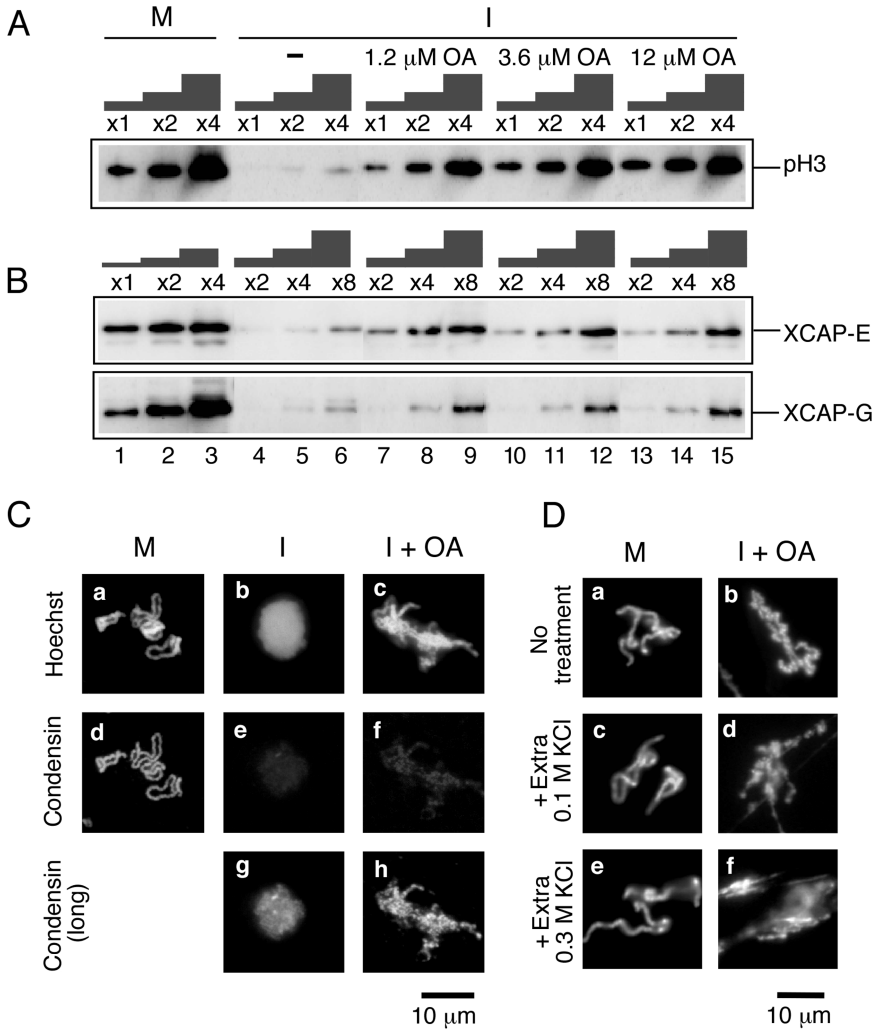

Figure 2. Stimulation of chromosomal binding of condensin I by OA in interphase extracts. **(A)** Sperm nuclei were incubated with mitotic extract (lanes 1–3), interphase extract (lanes 4–6), interphase extract supplemented with 1.2 (lanes 7–9), 3.6 (lanes 10–12) or 12 μM (lanes 13–15) of OA at 22°C for 2 h. Chromatin-bound proteins were dissolved with SDS-PAGE sample buffer, and 12.5 (lanes 1, 4, 7, 10 and 13), 25 (lanes 2, 5, 8, 11 and 14) and 50% (lanes 3, 6, 9, 12 and 15) of each sample were separated by SDS–PAGE, and immunoblotted with anti-phospho H3. **(B)** Samples were prepared as described in (A), and 6.25% (lane 1), 12.5% (lanes 2, 4, 7, 10 and 13), 25% (lanes 3, 5, 8, 11 and 14) and 50% (lanes 6, 9, 12 and 15) of samples were blotted using anti-XCAP-E (upper), and anti-XCAP-G (lower) antibodies. **(C)** Sperm chromatin was assembled in the mitotic extract (a, d), interphase extract (b, e, g), and interphase extract supplemented with 3.6 μM OA (c, f, h). Samples were fixed and stained with Hoechst (a, b, c; first low), and anti-XCAP-H antibody (d, e, f; second low, short exposure: g, h; third low, long exposure). Bar, 10 μm. **(D)** Sperm chromatin was assembled in the mitotic extract (a, c, e) or interphase extract supplemented with 3.6 μM OA (b, d, f). After assembly, the reaction mixtures were supplemented with the indicated extra concentration of KCl at 22°C for 20 min, fixed, and stained with Hoechst. Bar, 10 μm.

RNAi depletion of Glc7/PP1 resulted in the phosphorylation of Ser10 (36). Under this condition, a portion of condensin I subunits was targeted to the assembled chromatin: about 12.5% as much condensin I was associated with chromatin as in the mitotic extract (Figure 2B). Next, we examined the chromatin structure by Hoechst staining (Figure 2C, upper). When OA was supplemented into the interphase extract, sperm chromatin was converted into partially condensed individual chromatin fibers (Figure 2Cc), which are completely different from the round-shaped chromatin sphere in the interphase extract (Figure 2Cb). However, the chromatin fibers assembled in the OA-treated interphase extract were thinner and much more fragile than chromosomes assembled in a mitotic extract (Figure 2Ca). We also investigated the localization of condensin I by immunostaining using anti-XCAP-H antibody, because it is reported that condensin is bound to the specific regions of chromatin (Figure 3C, middle lower), such as rDNA and centromere (37,38), was regulated by Aurora B; however, only weak staining signal was observed on the chromosome-like structure assembled in the interphase extract supplemented with OA (Figure 2Cf). Moreover, no specific region was intensively stained even when the signal was enhanced by long exposure (Figure 2Ch).

Then, to compare the structural integrity of the chromosomes assembled in the OA-supplemented interphase extract with that assembled in the mitotic extract, the assembled structures were treated with buffers containing increasing concentrations of KCl (Figure 2D).

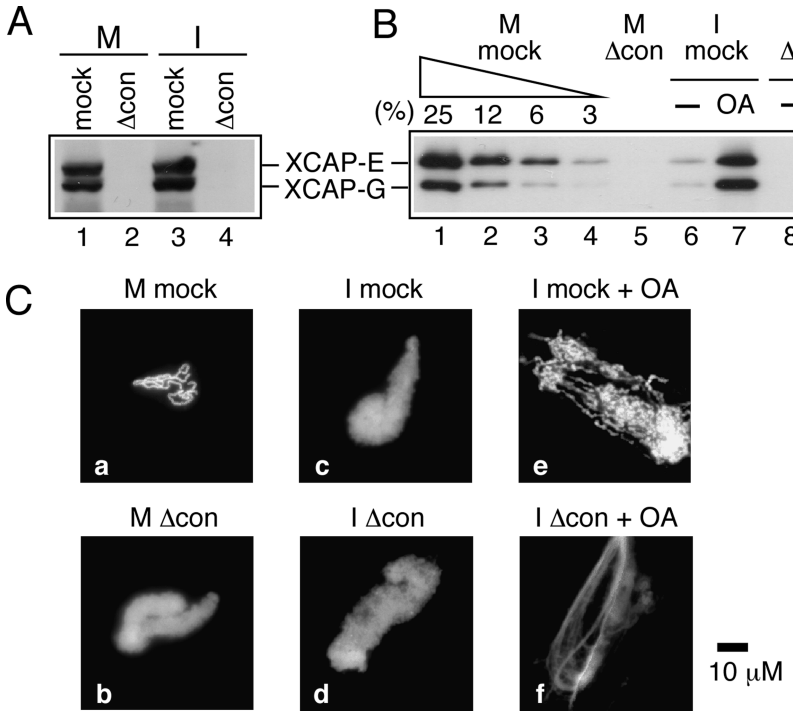

Figure 3. Requirement of condensin in OA-dependent partial chromosome condensation in the interphase extract. **(A)** Mitotic (lanes 1–2) and interphase (lanes 3–4) extracts were mock-depleted (lanes 1 and 3) or depleted with a mixture of affinity-purified condensin antibodies (lanes 2 and 4). Equal volumes of these extracts were subjected to SDS-PAGE, blotted, and detected using the indicated antibodies. **(B)** Sperm chromatin was incubated with mock-depleted mitotic extract (lanes 1–4), condensin-depleted mitotic extract (lane 5), mock-depleted interphase extract (lane 6), mock-depleted interphase extract supplemented with OA (lane 7), condensin-depleted interphase extract (lane 8) or condensin-depleted interphase extract supplemented with OA (lane 9) at 22°C for 2 h. The assembled structures were isolated, and 25 (lane 1), 12.5 (lane 2), 6.3 (lane 3), 3.1 (lane 4) and 100% (lanes 5–9) were analyzed by immunoblotting with the indicated antibodies. **(C)** Sperm chromatin was incubated with mitotic extract (a), condensin-depleted mitotic extract (b), mock-depleted interphase extract (c), mock-depleted interphase extract with OA (d), condensin-depleted interphase extract (e), or condensin-depleted interphase extract with OA (f) at 22°C for 2 h. After incubation, assembled chromatin structures were fixed and stained with Hoechst. Bar, 10 μm.

Salt treatment of the chromosomes assembled in the mitotic extract resulted in a more extended and loosened structure (Figure 2Dd and g); however, the structures of individual chromosome axes were still observed even when treated with buffer containing extra 300 mM KCl. On the other hand, the chromosome-like structures assembled in the OA-treated interphase extract were fragile and easily broken by salt treatment (Figure 2De and h), indicating that they were qualitatively distinct from rigid chromosomes assembled in the mitotic extract.

The next question is whether partial chromosome condensation in the OA-supplemented interphase extract requires condensin I: it is possible that other factors activated by OA were implicated in chromosome condensation (39). To test this possibility, OA was supplemented into condensin I-depleted *Xenopus* egg extracts, and the chromatin structure was fixed and stained with Hoechst (Figure 3). The efficiency of immunodepletion was examined by immunoblotting using anti-XCAP-E, and -G antibodies (Figure 3A). Next, we examined the amount of chromatin associated condensin I. Condensin I was absent from chromatin fraction assembled in the condensin I-depleted extract with OA (Figure 3B, lane 9), in contrast to substantial amount of condensin I was attached to chromosomes assembled in the OA-treated interphase extract (Figure 3B, lane 7). Interestingly, chromosome condensation did not take place in the condensin I-depleted extract when OA was supplemented into the extract; only thin chromatin fibers were observed (Figure 3Cf). Thus, it is likely that OA-dependent chromosome condensation depends on condensin I.

Aurora B is the kinase implicated in the partial association of condensin I and chromosome condensation

Many kinases are activated by the addition of OA in the absence of Cdc2. Therefore, the next issue is to identify the kinase(s) that induces Ser10 phosphorylation, partial chromosome association of condensin I, and partial chromosome condensation in the OA-treated interphase extract. It is also possible that residual Cdc2/cyclin B in the interphase extract may be activated by this treatment, thereby causing these events. To address these questions, OA is supplemented into the interphase extract that had been depleted of Aurora B, Aurora A or Cdc2 (Figure 4). The efficiency of immunodepletion was determined by quantitative immunoblotting (Figure 4A). When OA was supplemented into Aurora A- or Cdc2-depleted interphase extracts, Ser10 phosphorylation and chromatin association of condensin I subunits were indistinguishable from those in the undepleted extract supplemented with OA

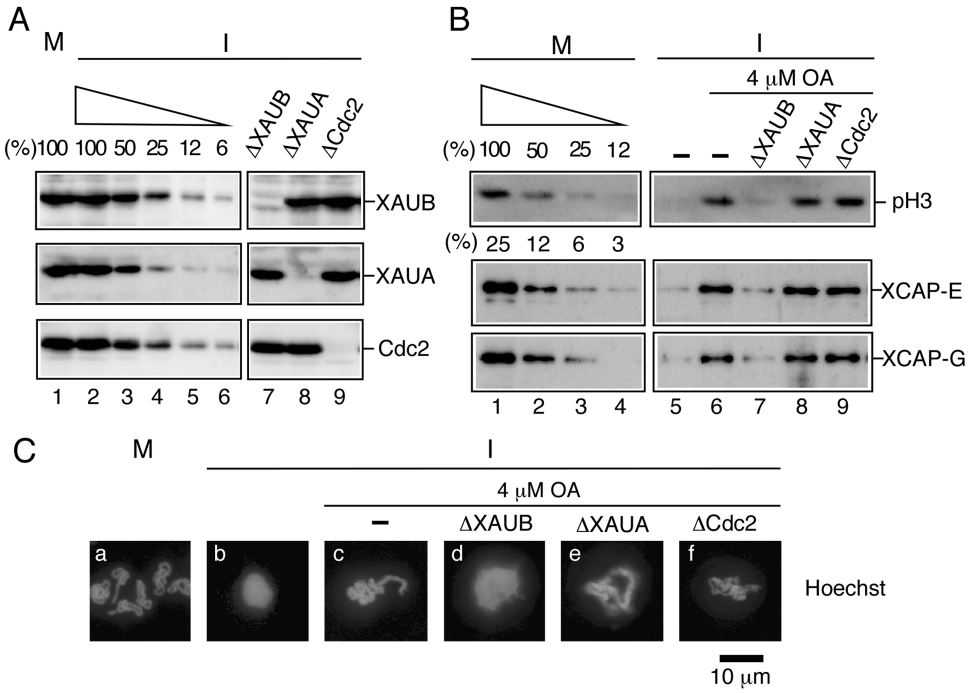

Figure 4. Requirement of Aurora B in the Cdc2-independent chromosomal targeting of condensin I. **(A)** Interphase extract was depleted with anti-Aurora B (lane 7), anti-Aurora A (lane 8) or anti-Cdc2 antibodies (lane 9). As a standard, 100% (lane 1) of mitotic extract, 100% (lane 2), 50% (lane 3), 25% (lane 4), 12.5% (lane 5) and 6.25% (lane 6) of interphase extract were loaded in parallel. Efficiency of immunodepletion was measured by quantitative immunoblotting using the antibodies indicated. **(B)** Sperm nuclei were incubated with mitotic extract (lanes 1–4), or interphase extract (lane 5), OA-treated interphase extract (lane 6), OA-treated interphase extract depleted of Aurora B (lane 7), OA-treated interphase extract depleted of Aurora A (lane 8), and OA-treated interphase extract depleted of Cdc2 (lane 9). Chromatin or chromosomes were isolated, and 100% (lanes 1, 5–9), 50% (lane 2), 25% (lane 3) and 12.5% (lane 4) of the samples were blotted by anti-phospho-H3 antibody (upper), anti-XCAP-C (middle) or anti-XCAP-E (lower) antibodies. In the case of blotting with anti-condensin subunit antibodies, a lower amount of samples was used as a standard, namely, 25% (lane 1), 12.5% (lane 2), 6.3% (lane 3) and 3.1% (lane 4). **(C)** Sperm chromatin was incubated with mitotic extract (a), interphase extract (b), OA-treated interphase extract (c), OA-treated interphase extract depleted of Aurora B (d), OA-treated interphase extract depleted of Aurora A (e) and OA-treated interphase extract depleted of Cdc2 (f). Samples were fixed and stained with Hoechst.

(Figure 4B, lanes 6, 8 and 9). Moreover, Aurora A or Cdc2 depletion did not interfere with the partial condensation of sperm chromatin in the presence of OA (Figure 4Cc, e and f). On the other hand, Ser10 phosphorylation and chromatin association of condensin I were almost abolished in the OA-treated interphase extract that had been depleted of Aurora B (Figure 4B, lane 7). Furthermore, the chromatin structure assembled in this extract was round (Figure 4Cd), similar to that assembled in the interphase extract (Figure 4Cb). From these results, it is strongly suggested that Aurora B is the corresponding kinase that brings about Ser10 phosphorylation, chromatin binding of condensin I and partial condensation of sperm chromatin.

Finally, we examined the supercoiling activity of condensin I in the OA-treated interphase extract because chromosome-like structures assembled in the extract were very fragile and totally differ from chromosomes assembled in the mitotic extract (Figure 5). Condensin I purified from the OA-treated interphase extract has low or undetectable supercoiling activity (Figure 5, upper, lanes 10–13), which was effectively the same as that of the inactive interphase condensin I (Figure 5, upper, lanes 6–9). DNA-binding activity of condensin I was almost constant under all conditions (Figure 5, lower).

DISCUSSION

Aurora B is the serine/threonine protein kinase that regulates many processes during cell division, such as chromosome condensation and cohesion, bipolar chromosome attachment, spindle checkpoint and cytokinesis (20,21). Aurora B phosphorylates histone H3 at Ser10, which has long been noted to be correlated with mitosis, both spatially and temporally. Ser 10 phosphorylation during mitosis is conserved in a wide range of eukaryotes, and is believed to be closely associated with the progression of mitosis, including chromosome condensation (40); however, recent studies have revealed that the relationship between Aurora B and chromosome condensation, or Ser10 phosphorylation and chromosome condensation is controversial (20,21).

In this study, we extensively analyzed the role of Aurora B during mitosis on chromosome condensation using a *Xenopus in vitro* system. We showed that Aurora B depletion from *Xenopus* egg extract did not affect the mitotic chromosome structure in the absence of Ser10 phosphorylation, consistent with previous reports (30,31). In contrast, the amount of condensin I associated with chromosomes decreased to 50% of that in the undepleted extract, by the depletion of Aurora B from mitotic egg extract. Furthermore, a substantial amount of condensin I

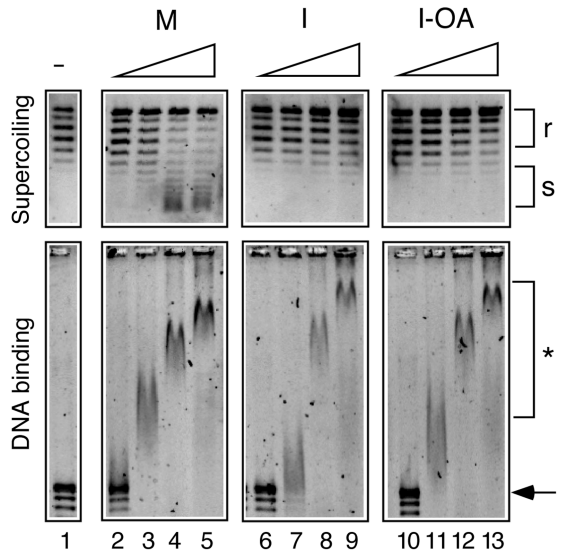

Figure 5. Effect of OA on the supercoiling activity of condensin I. Condensin I was affinity-purified from mitotic (lanes 2–5), interphase (lanes 6–9), or OA-treated interphase extract (lanes 10–13) and standard supercoiling (upper) and DNA binding assays (lower) were performed; r, relaxed circular DNA; s, positively supercoiled DNA; arrow, free DNA; asterisk, DNA bound to condensin I. The approximate molar ratios of condensin I to DNA were 15:1 (lanes 2, 6 and 10), 30:1 (lanes 3, 7 and 11), 60:1 (lanes 4, 8 and 12) and 120:1 (lanes 5, 9 and 13). Condensin I was omitted from lane 1.

(about 12.5% of mitotic level) was targeted to chromatin assembled in the interphase extract supplemented with OA, a PP1 and PP2A phosphatase inhibitor, followed by Ser10 phosphorylation. Significantly, the OA-dependent partial chromosomal association of condensin I was abolished by the depletion of Aurora B from the extract. From these results, we conclude that the OA-dependent chromosomal targeting of condensin I is Aurora B-dependent. It is reported that condensin binding to the centromere (37) or rDNA (38) was mediated by Aurora B/Ipl1; however, we did not find the specific staining signal of condensin I as detected by immunofluorescence study using XCAP-H antibody, when the interphase extract was treated with OA. Thus, the activation of Aurora B may be a prerequisite but is not sufficient for condensin I targeting to these chromatin regions. In fact, chromosomal binding of condensin I in the Aurora B-depleted mitotic extract, which contains Cdc2 in the absence of Aurora B, was 50%, and that in the OA-treated interphase extract, in which Aurora B was activated in the absence of Cdc2, was about 12.5%, compared to that in the mitotic extract, in which both Cdc2 and Aurora B were active. From these results, it is suggested that about 50% of the chromosomal targeting of condensin I is Cdc2-dependent, 12.5% of that is Aurora B-dependent, while the other 37.5% requires both Cdc2 and Aurora B. Our preliminary experiment showed that Aurora B depletion from mitotic extract resulted in the reduction of chromosome associated condensin II (data not shown); thus, the chromosomal targeting of condensin II might be also regulated by Aurora B. The regulation of the condensin II recruitment to chromosomes is an object of our next interest.

We considered three possibilities for the partial chromosomal targeting of condensin I induced by Aurora B. One is that Ser10 phosphorylation of nucleosomal H3 by Aurora B is implicated during the process (40). Phosphorylated Ser10 of H3 may act as a direct receptor for condensin I binding; for example, it is reported that condensin I is colocalized with phosphorylated H3 during the early stage of chromosome condensation (41). Very recently, it was proposed that H3 phosphorylation is required for the dissociation of HP1 during mitosis (42,43); therefore, it is possible that the local decondensation induced by HP1 dissociation could help condensin I to access chromatin DNA. The second possibility is that chromosomal Aurora B may act as a direct ligand for condensin I binding, or Aurora B could work as condensin I cargo to the chromosomes; however, we have not yet detected the interaction of condensin I and Aurora B. The third possibility is that Aurora B phosphorylates condensin I and phosphorylation may be involved in condensin I binding to chromosomes. Aurora B is able to phosphorylate condensin I *in vitro*. (data not shown). Alternatively, Aurora B may phosphorylate some proteins that induce the chromosomal targeting of condensin I, or Aurora B may inactivate inhibitors that prevent the chromosomal targeting of condensin I.

The next question is why chromosomes were normally condensed in the Aurora B-depleted mitotic extract, despite the amount of condensin I attached to chromosomes being reduced. We consider that discrepancy results from a large stockpile of protein components, which constitute the chromosome structure, such as condensin I in the *Xenopus* egg extract; therefore, the 50% reduction of chromosomal condensin I may not affect apparent chromosome condensation. In fact, mitotic chromosomes were assembled normally in the mitotic extract that was depleted of 75% of condensin I (data not shown). It is possible that the consequence of Aurora B depletion differs depending on the species, and the developmental stage may come from differences in the concentration of condensin I in the cells.

It is also noted that all of our experiments were performed using a high-speed supernatant, in which no DNA replication occurs, just to focus on the mechanism of the chromosomal association of condensin I. The use of a low-speed supernatant, as described by Cuvier and Hirano, may reflect the more physiological process for chromosome condensation because it allows one round of replication before mitosis (35). It was reported that chromosomal targeting of condensin I and chromosome condensation took place in a low-speed supernatant that was depleted of Aurora B (31) as well as in a high-speed supernatant (35). Precise analysis of the effect of Aurora B depletion on the amount of chromosome bound condensin I in a low-speed supernatant is the next interest of our work.

Finally, we considered two possibilities for OA-dependent partial chromosome condensation in the interphase extract: one is that condensin I bound to sperm chromatin drives chromosome condensation, and the other is that factors other than condensin I are

implicated in condensation. It is reported that the putative factor, RCA, is able to compact chromosome structures without condensin, and is inactivated by PP1 (39), which supports the latter model. But the former model is likely to account for the OA-dependent partial chromosome condensation in this experimental system because chromosome condensation was abolished when condensin I was depleted from OA-treated interphase extract. However, the chromosome-like structures assembled in the OA-treated interphase extract are very fragile and easily collapsed in the presence of high salt. It is noted that the supercoiling activity of condensin I purified from the extract was very weak, indistinguishable from that purified from the interphase extract (Figure 5). Thus, chromosome-like structures may be assembled in the absence of supercoiling activity of condensin I; however, the supercoiling activity of condensin I is required to give structural integrity to the assembled chromosomes. Alternatively, we cannot exclude the possibility that the amount of condensin I associated with chromosomes in the OA-treated interphase extract are too small to make rigid structures.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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