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Negative regulation of condensin I by CK2-mediated phosphorylation

Ai Takemoto[1,2,7], Keiji Kimura[1,3,4,7,*], Junn Yanagisawa[3], Shigeyuki Yokoyama[2,5] and Fumio Hanaoka[1,4,6,*]

[1]Cellular Physiology Laboratory, Discovery Research Institute, RIKEN, Wako, Saitama, Japan, [2]Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo, Japan, [3]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba Science City, Ibaraki, Japan, [4]Solution Oriented Research for Science and Technology (SORST) from the Japan Science and Technology Agency, Wako, Saitama, Japan, [5]RIKEN Genomic Sciences Center, Suehiro-cho, Tsurumi, Yokohama, Japan and [6]Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka, Japan

Condensin I, which plays an essential role in mitotic chromosome assembly and segregation *in vivo*, constrains positive supercoils into DNA in the presence of adenosine triphosphate *in vitro*. Condensin I is constitutively present in a phosphorylated form throughout the HeLa cell cycle, but the sites at which it is phosphorylated in interphase cells differ from those recognized by Cdc2 during mitosis. Immunodepletion, *in vitro* phosphorylation, and immunoblot analysis using a phospho-specific antibody suggested that the CK2 kinase is likely to be responsible for phosphorylation of condensin I during interphase. In contrast to the slight stimulatory effect of Cdc2-induced phosphorylation of condensin I on supercoiling, phosphorylation by CK2 reduced the supercoiling activity of condensin I. CK2-mediated phosphorylation of condensin I is spatially and temporally regulated in a manner different to that of Cdc2-mediated phosphorylation: CK2-dependent phosphorylation increases during interphase and decreases on chromosomes during mitosis. These findings are the first to demonstrate a negative regulatory mode for condensin I, a process that may influence chromatin structure during interphase and mitosis.

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Introduction

The packaging of long DNA molecules into compact rod-like structures characteristic of mitotic chromosomes ensures faithful segregation of genomic material during mitosis and meiosis. Failure of this process may lead to aneuploidy, cancer, and cell death. Despite its importance, the molecular bases underlying dynamic changes in higher-order chromosome structure during mitosis have remained enigmatic.

About a decade ago, studies using *Xenopus* egg extracts led to the identification of a five-subunit protein complex, 13S condensin, now referred to as condensin I, which plays a central role in the assembly and maintenance of mitotic chromosome structure (Hirano and Mitchison, 1994; Hirano *et al*, 1997). Condensin I is the main component of mitotic chromosomes (Saitoh *et al*, 1994; Hudson *et al*, 2003; Maeshima and Laemmli, 2003). The two core subunits of condensin I, CAP-C/SMC4 and CAP-E/SMC2, belong to the SMC (structural maintenance of chromosomes) family of adenosine triphosphatases (ATPases), which are involved in many aspects of chromosome dynamics (Jessberger, 2002; Losada and Hirano, 2005; Nasmyth and Haering, 2005; Uhlmann and Hopfner, 2006). The SMC2–SMC4 heterodimer adopts a V-shaped structure with an ATP-binding 'head' domain and Walker A and Walker B motifs at the distal ends of each arm (Melby *et al*, 1998; Anderson *et al*, 2002). It is thought that ATP binding and hydrolysis of each 'head' domain modulates engagement and disengagement of the 'head' domain, a cycle that is presumed to reconfigure DNA and chromatin structure. Purified SMC2–SMC4 heterodimers possess the ability to reanneal complementary single-stranded DNAs into double-stranded DNA in an ATP-independent manner (Sutani and Yanagida, 1997; Sakai *et al*, 2003). However, no ATP-dependent activities have been detected for the SMC heterodimer. The remaining three subunits (CAP-D2, -G and -H) are not related to SMC proteins and have possible roles in condensin regulation (Kimura and Hirano, 2000; Schleiffer *et al*, 2003). All five subunits of condensin I are highly conserved among eukaryotes ranging from yeast to the human (Hirano, 2000, 2005; Hagstrom and Meyer, 2003; Nasmyth and Haering, 2005).

The purified condensin I holo-complex constrains positive superhelical tension into DNA in an ATP-hydrolysis-dependent manner, as has been shown using supercoiling and knotting assays (Kimura and Hirano, 1997; Kimura *et al*, 1999; Hagstrom *et al*, 2002). This activity is stimulated by the Cdc2-dependent phosphorylation of the non-SMC subunits during mitosis, suggesting that this process may contribute to compaction of chromatin fibers during mitosis (Kimura *et al*, 1998, 2001; Hagstrom *et al*, 2002).

More recently, a second condensin complex, condensin II, which has the same two SMC subunits as condensin I but a different set of non-SMC subunits (CAP-D3, -G2 and -H2), was found in vertebrate cells (Ono *et al*, 2003; Yeong *et al*, 2003). Condensin II contributes to mitotic chromosome assembly in a manner distinct from that of condensin I (Ono *et al*, 2003; Hirota *et al*, 2004; Gerlich *et al*, 2006), but its biochemical activities are poorly characterized. Condensin II associates with chromatin throughout the cell cycle, whereas

*Corresponding authors. K Kimura, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tenno-dai, Tsukuba Science City, Ibaraki 305-8572, Japan. Tel.: +81 29 853 6632; Fax: +81 29 853 4605; E-mail: kekimura@sakura.cc.tsukuba.ac.jp or F Hanaoka, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamada-Oka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 7975; Fax: +81 6 6877 9382; E-mail: fhanaoka@fbs.osaka-u.ac.jp
[7]These authors contributed equally to this work

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condensin I targets mitotic chromosomes after nuclear envelope breakdown and dissociates from chromosomes during anaphase (Hirano, 2005). However, it has been reported that a small amount of condensin I persists within the nucleus during interphase (Schmiesing *et al*, 2000; Cabello *et al*, 2001). In addition to their mitotic functions, condensins I and II have been implicated in chromatin regulation during interphase. For example, condensin is required for transcriptional repression in *Saccharomyces cerevisiae* and *Drosophila* (Bhalla *et al*, 2002; Dej *et al*, 2004; Machin *et al*, 2004). In *S. pombe*, the condensin I subunit, Cnd2, is involved in the DNA checkpoint response and the DNA excision repair pathway (Aono *et al*, 2002). Human condensin I interacts with the PARP–1–XRCC1 complex and plays a role in the repair of DNA single-strand breaks (Heale *et al*, 2006).

We investigated the regulation of the human condensin I complex during interphase. We previously reported that human condensin I is phosphorylated throughout the cell cycle (Takemoto *et al*, 2004). However, the sites at which condensin I is phosphorylated in interphase cells differ from those recognized by Cdc2 during mitosis (Takemoto *et al*, 2004). Now, we have found that CK2 is the main kinase that phosphorylates condensin I during interphase. CK2-mediated phosphorylation during interphase greatly reduced its supercoiling activity, in contrast to the slight stimulatory effect of mitosis-specific phosphorylation by Cdc2. In this report, we describe the effect of CK2-mediated phosphorylation of condensin I on cell-cycle regulation and discuss its biological significance.

Results

Supercoiling activity of condensin I is suppressed by phosphorylation during interphase

We previously reported that condensin I is phosphorylated in logarithmically growing (log-phase) cells and in mitotic HeLa cells, but specific sites detected by the phospho-specific monoclonal antibody MPM-2 are phosphorylated only during mitosis (Takemoto *et al*, 2004). Very little is known about the effects of interphase phosphorylation on the functions of condensin I, whereas mitotic Cdc2-dependent phosphorylation stimulates the supercoiling activity of condensin I in *Xenopus* or human (Kimura *et al*, 1998, 2001) and is required for nuclear targeting of condensin I in *S. pombe* (Sutani *et al*, 1999). To characterize the roles of phosphorylation during interphase, we purified condensin I from log-phase cells (in which more than 95% cells were in interphase) and mitotic HeLa cells and assessed its phosphorylation status and the effects of phosphorylation on its biochemical activities.

We first compared the phosphorylation levels of the interphase and mitotic forms of condensin I using Pro-Q Diamond phosphoprotein gel stain, which detects overall phosphorylation (Schulenberg *et al*, 2003). Four of the five subunits (hCAP-C, -D2, -G, and -H) were phosphorylated in both forms of condensin I, and their phosphorylation levels were indistinguishable (Figure 1A). However, two-dimensional tryptic phosphopeptide mapping (Russo *et al*, 1992) revealed that, for each subunit, the sites that were phosphorylated during interphase differed from those that were phosphorylated during mitosis (Figure 1B).

To test the effect of interphase and mitotic phosphorylation on supercoiling activity, both forms of condensin I were dephosphorylated with lambda phosphatase (λ-PPase) (Figure 1A, lanes 4 and 8). Removal of phosphates from the interphase form of condensin I resulted in a great increase in its supercoiling activity (see Figure 1C, upper panel), whereas dephosphorylation of mitotic condensin I resulted in a slight decrease in supercoiling activity (Figure 1C, upper panel). In contrast, the DNA-binding activities of the interphase and mitotic forms of condensin I were almost unaffected by dephosphorylation (Figure 1C, lower panel).

To further investigate the effects of interphase phosphorylation, the supercoiling activity and phosphorylation status of condensin I were determined after incubation of dephosphorylated condensin I with an interphase *Xenopus* egg extract and subsequent repurification. This treatment resulted in a decrease in supercoiling activity to a level similar to that of interphase condensin I before dephosphorylation (Figure 1D) accompanied by subunit rephosphorylation. On the other hand, supercoiling activity was increased by treatment with a mitotic egg extract (data not shown). These results suggest that supercoiling activity is greatly suppressed by interphase-specific phosphorylation. This is the first demonstration of negative regulation of condensin I.

Condensin I is phosphorylated by CK2 during interphase

In an attempt to identify the kinase or kinases that phosphorylate and inactivate condensin I during interphase, 4,5,6,7-tetrabromobenzotriazole (TBB), an inhibitor of CK2, was added to the log-phase HeLa cell culture because CK2 is abundant in eukaryotic cells, is constitutively active during the cell cycle, and has a wide spectrum of substrates (Litchfield, 2003; Meggio and Pinna, 2003). Condensin I in log-phase HeLa cells was hardly phosphorylated in the presence of TBB (Figure 2A), suggesting that CK2 is a likely candidate for phosphorylation of condensin I during interphase.

To confirm that CK2 phosphorylates condensin I during interphase, we used a *Xenopus* egg extract because it faithfully reproduces cell cycle events and contains a large stockpile of structural proteins and regulatory factors. We first examined the effect of immunodepletion of CK2 from this extract. When dephosphorylated condensin I was incubated with an interphase egg extract, it was rephosphorylated to a level similar to that of the interphase complex, but it was almost unaffected by the CK2-depleted interphase extract (Figure 2B, lane 6). When purified CK2 was added to the CK2-depleted interphase extract, condensin I was rephosphorylated (Figure 2B, lane 7). In contrast, condensin I was phosphorylated in the mitotic extract depleted of CK2, even though the phosphorylation level was reduced (Figure 2B, lane 9). These results suggest that condensin I is phosphorylated by CK2 alone or by kinases activated by CK2 in the interphase extract, but is also phosphorylated by other kinases independent of CK2 in the mitotic extract, as has been reported for Cdc2 (Kimura *et al*, 1998, 2001), in addition to CK2.

As condensin I subunits have several consensus phosphorylation sites that are recognized by CK2, we examined whether purified CK2 could phosphorylate condensin I. Purified CK2 phosphorylated the hCAP-C, -D2, -G and -H subunits of dephosphorylated condensin I, but not the sites that were recognized by MPM-2, indicating that CK2 phosphorylates these subunits at sites other than those

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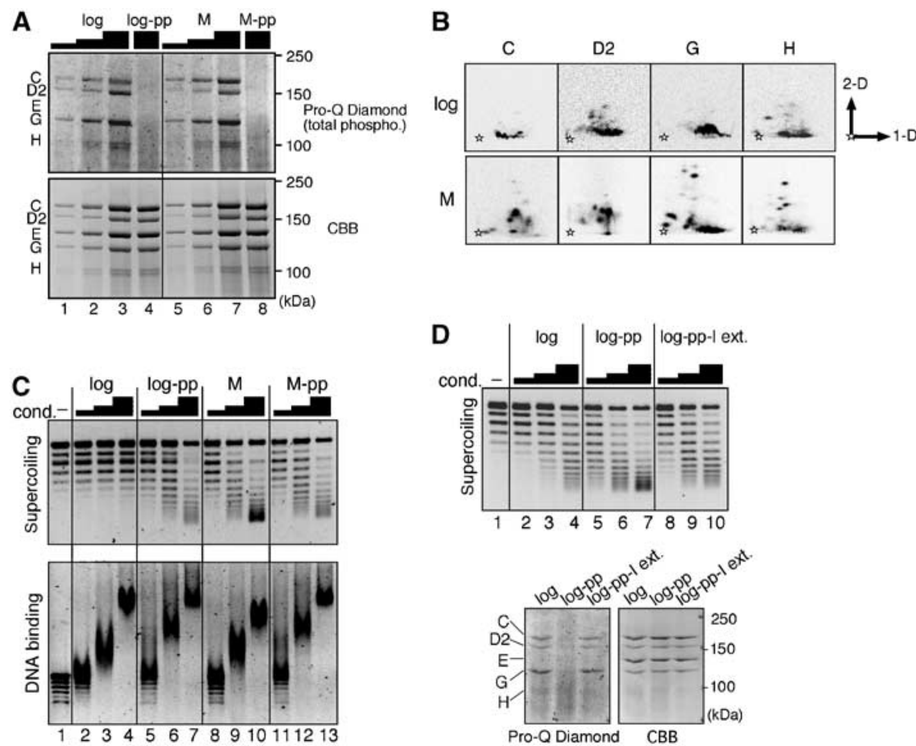

Figure 1 Negative regulation of condensin I by interphase phosphorylation. (**A**) Total phosphorylation levels. Condensin I was purified from log-phase (log, lanes 1–4) and mitotic HeLa cells (M, lanes 5–8) and treated with λ-PPase (log-pp, lane 4; M-pp, lane 8). Samples were resolved by SDS–PAGE followed by staining with Pro-Q Diamond phosphoprotein gel stain, which detects overall phosphorylation (Schulenberg *et al*, 2003). The levels of the hCAP-G subunit were 125 ng, lanes 1 and 5; 250 ng, lanes 2 and 6; 500 ng, lanes 3, 4, 7, and 8. (**B**) Phosphopeptide analysis of mitotic and log-phase condensin I. Condensin I was purified from ^{32}P-labeled log-phase cell extract (I, upper panel) or mitotic HeLa cell extract (M, lower panel). Phosphopeptide maps of the hCAP-C (C), hCAP-D2 (D2), hCAP-G (G), and hCAP-H (H) subunits are shown. **(C)** Supercoiling (upper panel) and DNA binding activities (lower panel) of log-phase (log, lanes 2–4) dephosphorylated log-phase (log-pp, lanes 5–7), M-phase (M, lanes 8–10), and dephosphorylated M-phase condensin I (M-pp, lanes 11–13). The supercoiling reactions, but not the DNA binding assay, were supplemented with topoisomerase I purified from HeLa cells. The molar ratios of condensin I to DNA in both reactions were 50:1 (lanes 2, 5, 8, and 11), 100:1 (lanes 3, 6, 9, and 12), or 200:1 (lanes 4, 7, 10, and 13). No protein was added to lane 1. The changes in the average linking number of the substrate DNA was measured to be +0.01 (lane 2), +0.05 (lane 3), +0.3 (lane 4), +0.6 (lane 5), +1.7 (lane 6), +4.1 (lane 7), +0.3 (lane 8), +2.3 (lane 9), +5.3 (lane 10), +0.2 (lane 11), +1.9 (lane 12), and +4.5 (lane 13). (**D**) Phosphorylation status and activity of condensin I following incubation in interphase extracts. Condensin I was purified from log-phase HeLa cells and treated with λ-PPase (lanes 5–10) or untreated (log, lanes 2–4). Condensin I treated with λ-PPase was incubated with buffer alone (log-pp, lanes 5–7) or with interphase *Xenopus* egg extract (log-pp-I ext., lanes 8–10) and repurified. The supercoiling activity of condensin I was assayed (upper panel). The molar ratios of condensin I to DNA were 100:1 (lanes 1, 4, and 7), 200:1 (lanes 2, 5, and 8), and 401:1 (lanes 3, 6, and 9). Protein was omitted from lane 1. The same condensin I samples (320 ng for hCAP-G) were subjected to SDS–PAGE and the gels were stained with Pro-Q Diamond (left lower panel) or Coomassie Brilliant Blue (right lower panel).

phosphorylated by Cdc2 (Figure 3A). Two-dimensional tryptic phosphopeptide mapping indicated that the some spots of the phosphopeptides (Figure 3B, dashed circle) of the hCAP-C, -D2, -G and -H subunits phosphorylated by CK2 resembled those labeled in log-phase cells (compare Figure 3B with Figure 1B, upper panel). In contrast, the hCAP-D2, -G and -H subunits of condensin I were phosphorylated by Cdc2, and the phosphopeptide patterns of the subunits phosphorylated by Cdc2 were similar to those labeled in mitotic cells (data not shown).

Next, we tested whether the supercoiling activity of condensin I was reduced by CK2 phosphorylation. When dephosphorylated condensin I was treated with CK2, its supercoiling activity was decreased to a level similar to that of interphase condensin I before dephosphorylation (Figure 3C). In contrast, the supercoiling activity of interphase condensin I was stimulated by Cdc2 treatment, but this activity was still weaker than that of the dephosphorylated form of condensin I (Figure 3C, lane 7). When dephosphorylated condensin I was phosphorylated by Cdc2, its supercoiling activity increased further (Figure 3C, lane 8) to a level similar to that of mitotic condensin I (data not shown), which suggests that both phosphorylation by Cdc2 and dephosphorylation of CK2-specific sites are required for full activation of condensin I. Taken together, we consider that some sites are labeled by CK2 both *in vivo* and *in vitro*, and these sites are implicated in the inactivation of condensin I during interphase.

To test further the role of CK2-mediated phosphorylation on the regulation of condensin I, dephosphorylated condensin I was treated by any combinations of CK2 and Cdc2 (Figure 3D). The Cdc2-mediated phosphorylation level was not reduced by CK2-mediated phosphorylation, as detected by immunoblotting using MPM-2 that recognizes phosphorylation of Cdk-consensus sequence (Figure 3D, upper). However, the stimulation of its supercoiling activity was weak when it was phosphorylated by CK2 and followed by Cdc2 (Figure 3D, lower, lanes 2 and 3). Alternatively, condensin I, which had been activated by Cdc2, was greatly inactivated by subsequent phosphorylation by CK2

(Figure 3D, lower, lanes 4 and 5). Thus, CK2-mediated phosphorylation reduces the supercoiling activity of condensin I in opposition to its stimulation by Cdc2. The similar results were obtained as to another Cdk kinase, Cdk2/CyclinA, that are active during interphase (Supplementary Figure 1).

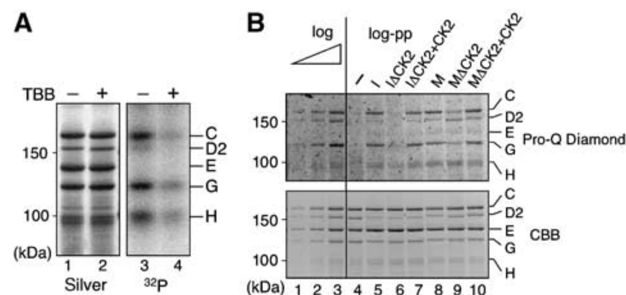

Figure 2 Phosphorylation of condensin I by CK2 during interphase. (**A**) Reduction of phosphorylation of condensin I in the presence of CK2 inhibitor (TBB). HeLa cells were cultured with ^{32}P-orthophosphate in the absence (lanes 1and 3) or presence (lanes 2 and 4) of 200 μM TBB. Condensin I was immunoprecipitated from these cell extracts, and the precipitated proteins were separated by SDS–PAGE and analyzed using silver staining (lanes 1 and 2) or an image analyzer (BAS-2500 Fuji Photofilm) (lanes 3 and 4). (**B**) Phosphorylation of condensin I by CK2 in interphase *Xenopus* egg extracts. λ-PPase-treated condensin I (log-pp, lanes 4–10) was incubated with buffer alone (−, lane 4), interphase *Xenopus* egg extract (I, lane 5), CK2-depleted interphase extract (IΔCK2, lane 6), CK2-depleted interphase extract supplemented with CK2 (IΔCK2 + CK2, lane 7), mitotic extract (M, lane 8), CK2-depleted mitotic extract (MΔCK2, lane 9), or CK2-depleted mitotic extract supplemented with CK2 (MΔCK2 + CK2, lane 10) and repurified. Samples and control condensin I purified from log-phase cells (I, lanes 1–3) were resolved by SDS–PAGE and the gel was stained with Pro-Q Diamond (upper panel) or Coomassie Brilliant Blue (lower panel).

Dephosphorylation of CK2 consensus sites is insufficient for mitotic chromosome condensation

We then determined whether the dephosphorylated form of condensin I could support chromosome condensation in interphase *Xenopus* egg extracts lacking Cdc2 activity because the supercoiling activity of dephosphorylated condensin I was almost as strong as that of the mitotic form (Figure 1C). To manipulate the phosphorylation level of condensin I in extracts, we tested several protocols for differentially immunodepleting and restoring purified condensin I. The efficiency of immunodepletion was monitored using immunoblotting (Figure 4A). We did not expect the CK2-specific sites of dephosphorylated condensin I to be phosphorylated after exposure to condensin- and CK2-depleted extracts.

When sperm chromatin was incubated in a control mitotic extract as a positive control, it was converted into a cluster of mitotic chromosomes (Figure 4B, panel 1). Chromosome condensation was not observed in condensin-depleted and condensin- and CK2-depleted mitotic extracts (Figure 4B, panels 2 and 5). When purified condensin I from HeLa cell extracts in either the interphase or dephosphorylated forms was included with condensin-depleted mitotic extracts, chromosome condensation was restored (Figure 4B, panels 3, 4, 6, and 7). Thus, CK2 depletion does not affect chromosome condensation in mitotic extracts. On the other hand, chromosome condensation did not occur in interphase extracts under any conditions (Figure 4B, panels 8–14), even when dephosphorylated condensin I was added to a condensin- and CK2-depleted extract in which condensin I was not phosphorylated (Figure 4B, panel 14). We also examined time-course experiments using ‘interphase extract that was depleted of condensin I, and added back log phase condensin I’, and ‘interphase extract that was depleted of CK2 and condensin, and added back dephosphorylated condensin I’; however,

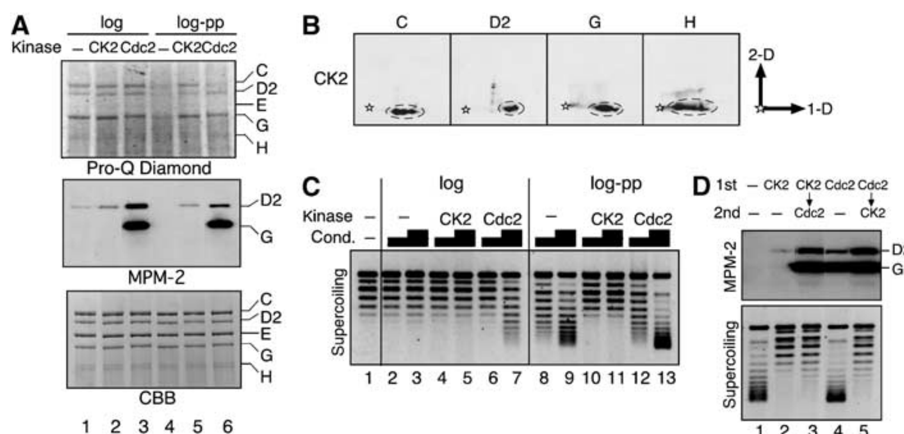

Figure 3 Inactivation of condensin I by CK2. (**A**) Phosphorylation of condensin I by CK2 and Cdc2. The interphase form of condensin I (log) or phosphatase-treated condensin (log-pp) was incubated with buffer alone (−, lanes 1 and 4), CK2 (CK2, lanes 2 and 5), or Cdc2 (Cdc2, lanes 3 and 6). Gels were stained with Pro-Q Diamond (upper panel), immunoblotted with the MPM-2 antibody (middle panel), and stained with Coomassie Brilliant Blue (lower panel). (**B**) Phosphopeptide analysis of CK2-labeled condensin I. Purified dephosphorylated condensin I was labeled by CK2 *in vitro*. Phosphopeptide maps of the hCAP-C (C), hCAP-D2 (D2), hCAP-G (G), and hCAP-H (H) subunits are shown. The phospho-spots that migrate similar to those of log phase condensin I are shown with dashed circle. (**C**) Inactivation of condensin I by CK2 and activation by Cdc2. A standard supercoiling assay was performed using the same condensin fractions as in (A). The approximate molar ratios of condensin I to DNA were 250:1 (lanes 2, 4, 6, 8, 10, and 12) and 500:1 (lanes 3, 5, 7, 9, 11, and 13). No protein was added to lane 1. (**D**) CK2-mediated phosphorylation did not affect Cdc2-mediated phosphorylation level, but inactivated condensin I. Dephosphorylated condensin I was incubated with buffer alone (lane 1), CK2 (lane 2), CK2 and then Cdc2 (lane 3), Cdc2 (lane 4), or Cdc2 and then CK2 (lane 5). An aliquot of each sample was immunoblotted with MPM-2 (upper panel). A supercoiling assay was performed using the same samples (lower panel). The approximate molar ratios of condensin I to DNA for the supercoiling assay 546:1.

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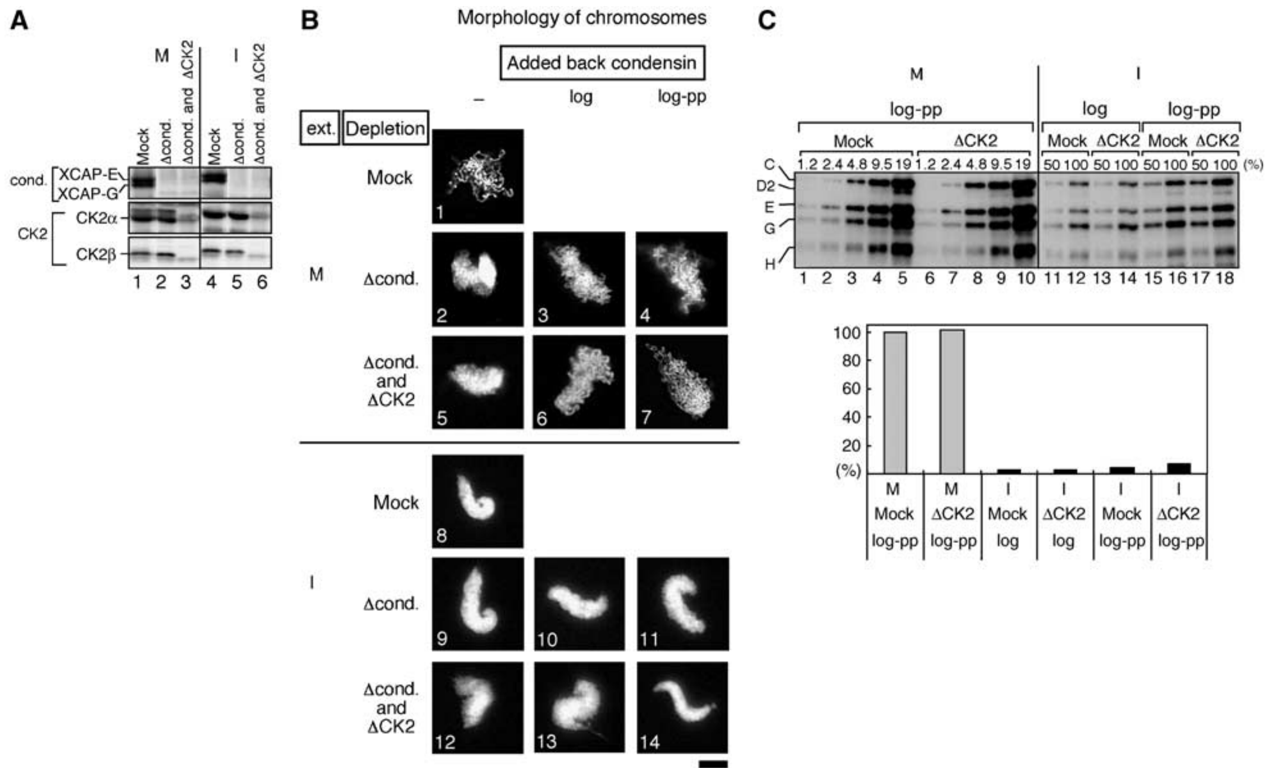

Figure 4 Effects of CK2 depletion on chromosome condensation and the chromosomal targeting of condensin. (**A**) Efficiency of immunodepletion. Mitotic extracts (M, lanes 1–3) or interphase extracts (I, lanes 4–6) were immunodepleted with control IgG (mock, lanes 1 and 4), with anti-XCAP-E and anti-XCAP-G antibodies (Δcond., lanes 2 and 5), or with a mixture of anti-XCAP-E, anti-XCAP-G, and anti-CK2β antibodies (Δcond. and ΔCK2, lanes 3 and 6). Equal volumes of each extract were analyzed by immunoblotting with the indicated antibodies. (**B**) Chromosomal morphology. Mitotic extracts were mock-depleted (panel 1), depleted of condensin I (panels 2–4), or depleted of condensin I and CK2 (panels 5–7). Interphase extracts were mock-depleted (panel 8), depleted of condensin I (panels 9–11), or condensin I and CK2 (panels 12–14). Log-phase condensin I (panels 3, 6, 10, and 13) or dephosphorylated condensin I (panels 4, 7, 11, and 14) was restored to the depleted extracts. Sperm DNA was incubated with these extracts for 2 h, and chromosomal morphology was observed after fixation followed by staining with Hoechst dye (bar: 10 μm). (**C**) Quantification of condensin I in chromatin-bound fractions. Log-phase condensin I was added to condensin I-depleted interphase extracts (lanes 1–2) or to CK2- and condensin I-depleted interphase extracts (lanes 3–4). Dephosphorylated condensin I was added to condensin I-depleted interphase extracts (lanes 5–6), CK2- and condensin I-depleted interphase extracts (lanes 7–8), condensin I-depleted mitotic extracts (lanes 9–13), or CK2- and condensin I-depleted mitotic extracts (lanes 14–18). Sperm chromatin was incubated with each extract at 22°C for 2 h, chromatin was isolated, and 50% (lanes 1, 3, 5, and 7), 100% (lanes 2, 4, 6, and 8), 1.2% (lanes 9 and 14), 2.4% (lanes 10 and 15), 4.8% (lanes 11 and 16), 9.5% (lanes 12 and 17), and 19% (lanes 13 and 18) of the bound proteins were analyzed by quantitative immunoblotting (upper panel). The amounts of the hCAP-E subunit bound to sperm chromatin were determined using the Las 1000 imaging system (Fujifilm). The amount of chromosome-bound hCAP-E in each extract relative to that in the mock-depleted mitotic extract is indicated as a percentage (lower graph).

chromosome condensation did not occur at any time points (Supplementary Figure 2). Therefore, we examined the association of condensin I with chromatin using quantitative immunoblotting. The amount of condensin I bound to sperm chromatin in interphase extracts was <10% of that of mitotic extracts under all conditions (Figure 4C). Depletion of Cdc2 from mitotic extract also resulted in the great reduction of chromosomal binding of condensin I (Supplementary Figure 3).

These results suggest that Cdc2 activity is required for most chromosomal binding activity of condensin I in *Xenopus* egg extract. Therefore, sperm chromatin is not converted into mitotic chromosome-like structures in interphase extracts that lack Cdc2 activity, even when the supercoiling activity is stimulated by dephosphorylation. However, when dephosphorylated condensin I was included in a CK2-depleted interphase extract, the amount of condensin I targeted to sperm chromatin was more than twice that observed when its phosphorylated interphase form was included (Figure 4C). Moreover, the supercoiling activity of dephosphorylated condensin I was more than four times stronger than that of interphase condensin I. Thus, it is tempting to speculate that CK2-dependent phosphorylation of condensin I may control chromatin structure and function during interphase by regulating the chromatin-binding and supercoiling activities of condensin I.

The CK2-specific consensus sites in condensin I are dephosphorylated on mitotic chromosomes

To determine if the CK2-specific consensus sites in condensin I subunits are phosphorylated in HeLa cells, we synthesized phosphopeptides containing single phosphoserine or phosphothreonine residues and prepared phospho-specific antibodies. Among them, an antibody against the hCAP-H sequence (PGLQAADphosphoS^{570}DDEDLDD) was available for detection of phospho-epitopes. The affinity-purified anti-phospho-hCAP-H antibody recognized hCAP-H prepared from log-phase cells (Figure 5A). Phosphorylation-specific signals were blocked by the corresponding antigen peptide, but not by an unphosphorylated peptide of the same

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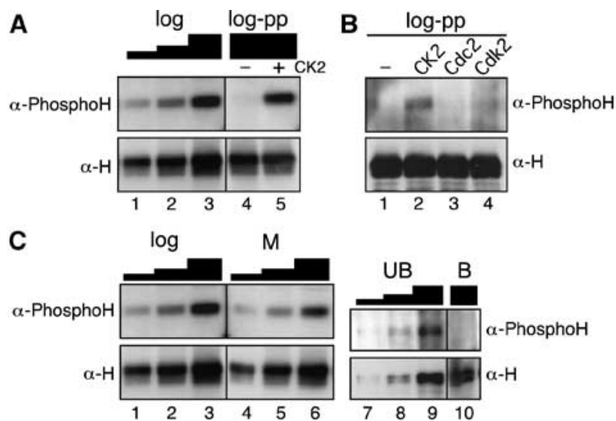

Figure 5 Fluctuation of CK2-dependent phosphorylation *in vivo*. (**A**) Recognition of a CK2-dependent phosphorylation site by a phospho-specific antibody. Condensin I was purified from log-phase cells (lanes 1–3), treated with λ-PPase (lanes 4 and 5), and phosphorylated by CK2 (lane 5). One hundred nanograms (lane 1), 200 ng (lanes 2, 4, and 5), or 400 ng (lane 3) of each sample was immunoblotted using the antiphospho-H (upper) or anti-hCAP-H antibodies (lower). (**B**) Specificity of the phospho-specific antibody. Dephosphorylated condensin I (300 ng) was incubated with buffer alone (lane 1), CK2 (lane 2), Cdc2 (lane 3), or Cdk2/CyclinA (lane 4) at 30°C for 60 min, then immunoblotted with the indicated antibodies. (**C**) CK2-dependent phosphorylation in HeLa cells. Increasing amounts of condensin I, purified from log-phase cell extract (log, lanes 1–3) or mitotic cell extract (M, lanes 4–6), were immunoblotted using antibodies as indicated. The amounts of the hCAP-E subunit were 100 ng (lanes 1 and 4), 200 ng (lanes 2 and 5), and 400 ng (lanes 3 and 6). Condensin I was purified from the chromosome-unbound fraction (UB, lanes 7–9) or the chromosome-bound fraction (B, lane 10) of mitotic HeLa cells and analyzed by immunoblotting using the indicated antibodies. The relative levels of condensin I were 1:2:4:4 (lane 7:lane 8:lane 9:lane 10).

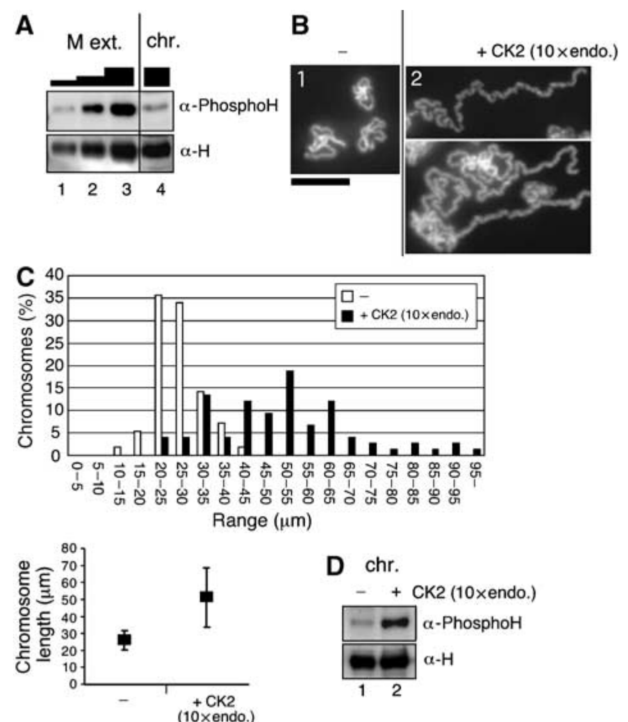

Figure 6 Dephosphorylation of CK2 sites required for chromosome condensation. (**A**) CK2-dependent phosphorylation in *Xenopus* egg extracts. Increasing amounts of condensin I purified from a mitotic *Xenopus* egg extract (lanes 1–3) or condensin I associated with sperm chromatin (lane 4) were immunoblotted with the indicated antibodies. The relative levels of condensin I were 1:2:4:4 (lane 1:lane 2:lane 3:lane 4). (**B**) Effect of CK2 on chromosome structure and phosphorylation status of chromosome-bound condensin I. Sperm chromatin was incubated with mitotic extract supplemented with buffer alone (–, panel 1) or a 10-fold excess of purified CK2 relative to endogenous CK2 (+CK2, panel 2) for 2 h and stained with Hoechst dye (bar: 10 μm). (**C**) Quantification of chromosome lengths. Distribution of chromosome length in control and CK2-supplemented extract was shown in the left graph (control, $n = 56$; CK2-supplemented, $n = 75$). The average length and standard error were shown in the right graph (control extract, 26.5 ± 5.6 μm; CK2-supplemented extract, 51.6 ± 17.3 μm). (**D**) Sperm chromatin was incubated with each extract as in (B); chromatin was then isolated and the bound proteins were analyzed by immunoblotting.

sequence (data not shown). λ-PPase treatment resulted in a loss of phospho-epitopes, and incubation of dephosphorylated condensin I with purified CK2 resulted in phosphorylation of the epitopes (Figure 5A). When dephosphorylated condensin I was treated with Cdc2 or Cdk2/CyclinA, this site was not phosphorylated (Figure 5B). Thus, it is likely that this phosphorylation site is specific for CK2.

We tested the phosphorylation level of this CK2-specific site of condensin I purified from log-phase or mitotic cells by immunoblotting using the antiphospho-hCAP-H antibody. The phosphorylation-specific signal of the mitotic form of condensin I was about half as strong as that of interphase condensin I (Figure 5C). As a portion of condensin I is associated with chromosomes in mitotic cells (Schmiesing *et al*, 2000; Takemoto *et al*, 2004), we compared the extent of phosphorylation of this site in chromosome-bound and -unbound condensin I in mitotic cells. The phosphorylation-specific signal of condensin I associated with chromosomes was much weaker than that of chromosome-unbound condensin I in mitotic cells (Figure 5D). This regulation is in contrast to that by Cdc2; chromosome-bound condensin I has stronger phospho-epitopes to MPM-2, which recognizes the Cdc2-phosphorylation sites, than chromosome-unbound form (Takemoto *et al*, 2004). As a positive control for subcellular fractionation, we carried out immunoblot analyses with antibodies against histone H3 and lamin B1 (data not shown). In contrast, the CK2-mediated phospho-signal of condensin I purified from chromatin fraction was comparable to that of chromatin-unbound condensin I in log-phase cells (Supplementary Figure 4). Thus, this CK2-mediated site was dephosphorylated efficiently only on mitotic chromosomes.

Because the CK2-specific phosphorylation site present in the hCAP-H subunit is also conserved in *Xenopus*, we determined whether this site is also phosphorylated in *Xenopus* egg extracts. The phospho-hCAP-H-specific antibody also recognized phosphorylation of the corresponding site of the XCAP-H subunit of *Xenopus* condensin I. Sperm chromatin was incubated in a mitotic *Xenopus* egg extract, and the CK2 phosphorylation status of chromatin-bound and chromatin-unbound condensin I was examined. Interestingly, the phosphorylation level of the CK2 site of chromatin-bound condensin I was also much lower than that of the chromatin-unbound form in the mitotic *Xenopus* egg extract (Figure 6A). When purified human condensin I was supplemented in the condensin I-depleted mitotic *Xenopus* egg extract (Figure 4B), the CK2 site was also efficiently dephosphorylated (Supplementary Figure 5).

Taken together, these results suggest that the dephosphorylation of CK2-specific phosphorylation sites on chromosomes

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may be a mitotic regulatory mechanism. Because dephosphorylation of CK2-specific phosphorylation sites greatly increases the supercoiling activity of condensin I, this regulatory process may be required for mitotic chromosome condensation.

To test this hypothesis, a 10-fold excess of purified CK2 (relative to the endogenous level) was added to a mitotic extract. The chromosomes that assembled in the CK2-supplemented mitotic extract were longer, thinner, and more partially condensed than those that assembled in a normal mitotic extract (Figure 6B). To characterize and quantify the observed morphological defect caused by CK2-addition, the length of the chromosomes assembled in the control and CK2-supplemented extracts was measured as described (Maresca *et al*, 2005). On average, chromosome length in the CK2-supplemented extract was about two-fold longer than in the control extract, indicating that chromosomes assembled in the CK2-supplemented extract were less condensed (Figure 6C). This effect was accompanied by an increase in the extent of phosphorylation of the CK2 site (Figure 6D). Quantitative immunoblotting indicated that about 50% of the condensin I on chromosomes assembled in the CK2-supplemented extract was phosphorylated (Supplementary Figure 6). Time-course experiment showed that the chromosomes assembled in the CK2-supplemented extract exhibited morphological defects that were characterized by elongated chromosomes over the time, in contrast to the sperm chromatin being converted to compacted chromosomes within 2 h in the normal mitotic extract (–CK2) (Supplementary Figure 7). All condensin I and II subunits were properly targeted to the aberrant chromosomes (data not shown).

These results suggest that, in addition to phosphorylation mediated by Cdc2, dephosphorylation of the CK2-specific sites of condensin I may be required for complete condensation of mitotic chromosomes.

Discussion

Condensin I, a key protein that contributes to mitotic chromosome condensation *in vivo*, is constitutively present throughout the cell cycle. However, chromatin is relaxed during interphase and is converted into compact rod-like structures (chromosomes) over a short period during mitosis. Thus, the function of condensin I must be tightly regulated during the cell cycle. The enzymatic activities of condensin I are positively regulated by Cdc2-induced mitosis-specific phosphorylation (Kimura *et al*, 1998, 1999, 2001; Hagstrom *et al*, 2002). In this study, we demonstrated that the negative regulatory mode of condensin I is induced by CK2-mediated phosphorylation and that the positive regulatory mode of condensin I is induced by mitosis-specific Cdc2-mediated phosphorylation. An updated model for the regulation of condensin I during the cell cycle is shown in Figure 7. Translated condensin I is phosphorylated and inactivated by CK2 in interphase cells. At the onset of mitosis, condensin I is partially activated by Cdc2-mediated phosphorylation and targeted to mitotic chromosomes, where it is activated further by dephosphorylation of CK2-specific sites. Alternatively, it is also possible that the CK2 sites of condensin I was dephosphorylated in the cytosol and this form binds and functions on chromosomes (dashed line). Thus, together with Cdc2, CK2 may be a key kinase that regulates condensin I activity.

This is consistent with a previous report, in which the interphase form of condensin I was shown to be activated by Cdc2-mediated phosphorylation, but the activity was still weaker than that of mitotic form (Kimura *et al*, 1999). It is possible that other mitotic kinases might be required for the

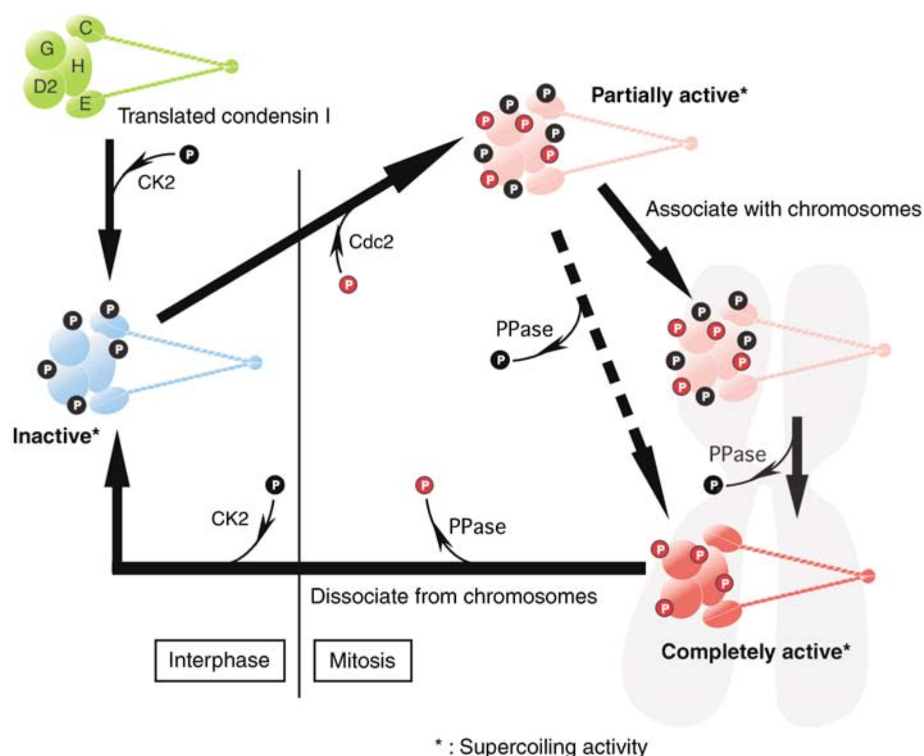

Figure 7 A model for the regulation of condensin I by phosphorylation with CK2 and Cdc2. See the text for details.

complete activation of condensin I; however, we propose here an alternative model in which dephosphorylation of some sites is important. The result, in which dephosphorylation of mitotic condensin I has a subtle effect on its supercoiling activity, looks conflict with the previous report (Kimura *et al*, 1998). We consider the reason for the discrepancy is that the involvement of Cdc2- and CK2-mediated phosphorylation in condensin I activity may vary during the development of an organism and between different organisms. When condensin I purified from mitotic HeLa cells is dephosphorylated, the reduction of supercoiling activity of condensin I by the dephosphorylation of Cdc2-mediated sites may be offset by the stimulation by the dephosphorylation of CK2-mediated sites; therefore, there is a little change in its activity. Whereas, the effect of Cdc2 may be more dominant in the *Xenopus* embryonic system, because the Cdc2-dependent phosphorylation sites are almost fully phosphorylated in *Xenopus* mitotic egg extract (data not shown). The phosphatase(s) that dephosphorylate CK2-mediated phosphorylation sites of condensin I on mitotic chromosomes remain to be identified. Okadaic acid, an inhibitor of type 1 and type 2A phosphatase, did not affect the phosphorylation level of the CK2-dependent site in hCAP-H attached to mitotic chromosomes (data not shown), suggesting that other types of phosphatase are implicated in this process.

In this study, we demonstrated that condensin I is negatively regulated by CK2. What is the physiological significance of the inhibition of condensin I during interphase? We consider two models: one is that it affects chromatin structure; the other is that it influences interaction of condensin I with other proteins. It is possible that CK2-mediated phosphorylation contributes to the relaxation of chromatin because condensin I compacts chromatin during mitosis (Figure 7). It is believed that cellular processes, including transcription, repair, and replication, are tightly regulated by chromatin structure. Recently, several lines of evidence have indicated that condensins, in addition to their mitotic functions, play critical roles in interphase. Condensin I is required for gene silencing in *S. cerevisiae* and *Drosophila* (Bhalla *et al*, 2002; Dej *et al*, 2004; Machin *et al*, 2004). Condensin I is required for DNA repair and the DNA damage checkpoint response in *S. pombe* (Aono *et al*, 2002). Dosage-compensation factors, which resemble the condensin complex, bind hermaphrodite X chromosomes and reduce gene expression in *Caenorhabditis elegans* (Chuang *et al*, 1994; Lieb *et al*, 1998; Hagstrom and Meyer, 2003). Interestingly, condensin negatively regulates gene expression via transcriptional control in many species. Because condensin I has activity to constrain DNA supercoiling and looping *in vitro* (Kimura and Hirano, 1997; Bazett-Jones *et al*, 2002), it is possible that this activity is required to some extent for compacting chromatin during interphase and for repressing transcription. If so, it is tempting to speculate that CK2-dependent phosphorylation of condensin I may relax the chromatin structure and stimulate transcription by suppressing supercoiling activity. Recently, it was reported that condensin I interacts with other proteins. For example, condensin I interacts with DNA methyltransferase (Geiman *et al*, 2004). The CAP-G subunit of condensin I interacts with protein phosphatase 2A and is related to gene bookmarking (Xing *et al*, 2005). In *Drosophila* cells, the Barren (CAP-H) subunit of condensin I and DNA topoisomerase II colocalize with Polycomb proteins and maintain gene silencing (Lupo *et al*, 2001). More recently, human condensin I was reported to interact with the poly(ADP-ribose) polymerase I and XRCC1 complex, and was implicated in the single-strand-break repair pathway (Heale *et al*, 2006). Thus, it is also possible CK2-mediated phosphorylation regulates the interaction of condensin I with other proteins and influences several chromatin functions.

Condensin I is abundant throughout the cell cycle, but exhibits dynamic cell-cycle-specific subcellular localization (Schmiesing *et al*, 2000; Cabello *et al*, 2001; Takemoto *et al*, 2004). Although a considerable amount of condensin I associates with mitotic chromosomes, most condensin I is present in the cytoplasm during interphase. Therefore, the concept that condensin I must be inactivated by the CK2-mediated phosphorylation may be questioned. However, a subpopulation of condensin I is present in nuclei (Schmiesing *et al*, 2000; Cabello *et al*, 2001), suggesting that condensin I may play a role during interphase. This possibility is supported by evidence that condensin I has interphase roles, such as DNA damage checkpoint response, DNA excision repair, DNA single-strand break repair, and transcriptional repression (Lupo *et al*, 2001; Aono *et al*, 2002; Bhalla *et al*, 2002; Dej *et al*, 2004; Heale *et al*, 2006). Thus, it is possible that the small amount of condensin I present in nuclei is sufficient for its interphase functions and that CK2-mediated phosphorylation regulates these functions. In contrast to condensin I, condensin II is present in nuclei throughout the cell cycle, although the total protein level of condensin II is lower than that of condensin I (Ono *et al*, 2003, 2004; Yeong *et al*, 2003; Hirota *et al*, 2004). Further elucidation of the regulation of condensin II during interphase is warranted as its role may be more important than that of condensin I during this period. Our findings concerning the interphase modification of condensin I provide a framework for such studies.

Finally, it would be important to elucidate the physiological significance of the CK2-mediated-phosphorylation in the regulation of the chromatin structure and functions. To this end, identifying and mutagenesis of the physiological relevant targets of CK2 may be the best procedures. However, the work is difficult and will take a lot of time, because condensin I is very large protein complex (670 kDa) and contains a lot of CK2-consensus sites, and moreover, expression and purification of the recombinant condensin I, especially *Xenopus* one, are difficult. We consider it as a long-term goal.

Materials and methods

Antibodies

Rabbit polyclonal antisera were raised against synthetic peptides corresponding to the N-terminal sequences of CK2α (MSGPVPSRA RVYTDC), the C-terminal sequence of *Xenopus* CK2β (CASNFKSPV KTMR), and human CK2β (CASNFKSPVKTIR), as described previously (Hirano *et al*, 1997; Kimura and Hirano, 1997). For phospho-specific antibodies, phosphopeptides bearing the CK2-specific consensus sequences of the hCAP-C, -D2, -G, and -H subunits and the corresponding unmodified peptides were synthesized. The sequences of the phosphopeptides were as follows: CTEKEIKDphosphoTEKEVDDL for hCAP-C, CVFSSDESphospho-SEEDLSAE for hCAP-D2, CTAEADSEphosphoSDHEVPEP for hCAP-G, and CPGLQAADphosphoSDDEDLDD for hCAP-H. Phospho-specific antibodies were affinity purified as described (Kimura *et al*, 1998). Among them, a phospho-specific antibody against the

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hCAP-H subunit was available for detecting CK2-dependent phosphorylation.

Cell culture

HeLa S3 cells were cultured in suspension at 37°C in Dulbecco's modified Eagle's minimal essential medium containing 100 U/ml penicillin G potassium, 100 mg/ml streptomycin sulfate, and 5% calf serum. Mitotic cells were collected by adding 150 ng/ml TN-16 (Wako) to logarithmically growing HeLa S3 cells (3×10^5 cells/ml) and incubating for 24 h, as described previously (Takemoto *et al*, 2004). ^{32}P-Labeling was performed as described (Takemoto *et al*, 2004).

Supercoiling and DNA-binding assays

The supercoiling assay was performed as described elsewhere (Takemoto *et al*, 2004). For quantification, the relative amount of each topoisomer was measured by LAS-1000 (FYJIFILM), and the mean superhelical tension was calculated. The DNA-binding assay was conducted as described previously (Kimura and Hirano, 1997).

Phosphorylation of dephosphorylated condensin I in Xenopus egg extracts

Dephosphorylated condensin I was incubated with 100 μl interphase or mitotic *Xenopus* egg extract at 22°C for 30 min and purified using the anti-hCAP-G antibody coupled to protein A Sepharose beads, as described for purification of condensin I.

Detection of total phosphorylation

The total phosphorylation level of condensin I was determined using Pro-Q Diamond phosphoprotein gel stain (Molecular Probes) as described (Schulenberg *et al*, 2003).

Immunodepletion and rescue

Condensin I was immunodepleted from *Xenopus* egg extracts as previously described (Hirano *et al*, 1997; Kimura and Hirano, 2000). For CK2 immunodepletion, 150 μg affinity-purified anti-CK2α and 65 μg anti-CK2β was incubated with 10 μl protein A Sepharose beads (Amersham Biosciences) for 1 h. The antibody-coupled beads were washed with XBE2 (50 mM HEPES (pH 7.7), 10 mM EGTA, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1.7% sucrose), 100 μl *Xenopus* egg extracts was added, and the mixtures were incubated on a rotating wheel at 4°C for 1 h. The supernatants were recovered and incubated with a fresh batch of the same beads. After 1-h incubation, the supernatants were recovered and used as depleted extracts. For rescue, purified condensin I or the CK2 fraction was prepared in XBE2 with 5 mM dithiothreitol and added to the depleted extracts at the same concentration as for the endogenous protein.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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