[Cell Cycle 3:5, 638-647; May 2004]; ©2004 Landes Bioscience

Report

Identification of Toposome, a Novel Multisubunit Complex Containing Topoisomerase IIα

Chee-Gun Lee*
Lisa K. Hague
Hong Li
Robert Donnelly

Department of Biochemistry and Molecular Biology; Graduate School of Biomedical Sciences; University of Medicine and Dentistry of New Jersey; Newark, New Jersey USA

*Correspondence to: Chee-Gun Lee; Department of Biochemistry and Molecular Biology; Graduate School of Biomedical Sciences; University of Medicine and Dentistry of New Jersey; 185 South Orange Avenue; Newark, New Jersey 07013 USA; Tel: 973.972.0130; Fax: 973.972.5594; E-mail: leecg@umdnj.edu

Received 03/03/04; Accepted 03/04/04

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=825

KEY WORDS

topoisomerase IIα, RNA helicase A, SRPK1, decatenation, knotting, mitosis

ACKNOWLEDGEMENTS

We thank Drs. Mukund Modak and Andrew Parrott for their comments and critical reading of the manuscript. We also thank Dr. Gideon Dreyfuss for providing antibody to hnRNP C (4F4), Dr. Maria M. Konarska for antibody to PRP8, Dr. Stephen J. Lippard for antibody to SSRP1, and Dr. Jerard Hurwitz for antibody to RHII/Gu.

This study was supported by a Research Grant from the University of Medicine and Dentistry of New Jersey (UMDNJ) Foundation to C.G. Lee.

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ABSTRACT

Topoisomerase IIα plays essential roles in chromosome segregation. However, it is not well understood how topoisomerase IIα exerts its function during mitosis. In this report, we find that topoisomerase IIα forms a multisubunit complex, named toposome, containing two ATPase/helicase proteins (RNA helicase A and RHII/Gu), one serine/threonine protein kinase (SRPK1), one HMG protein (SSRP1), and two pre-mRNA splicing factors (PRP8 and hnRNP C). Toposome separates entangled circular chromatin DNA about fourfold more efficiently than topoisomerase IIα. Interestingly, this decatenation reaction yields knotted circles, which are not seen in reactions provided with monomeric circular DNA. Our results also show that interaction among toposome-associated proteins is highest in G_2/M phase but drastically diminishes in G_1/S phase. These results suggest that toposome is a dynamic complex whose assembly or activation is subject to cell cycle regulation.

INTRODUCTION

For simultaneous separation at the metaphase-anaphase transition, sister chromatids undergo progressive structural changes. A multisubunit complex, cohesin, provides the physical linkage between the two sister chromatids as they are replicated in S phase. In *S. cerevisiae*, the cohesin complex consists of two chromosomal ATPases, Smc1 and Smc3, Scc1/Mcd1, and Scc3.[1,2] Several factors are required for loading cohesin onto replicating chromatin DNA, including the Scc2/Scc4 complex,[3] Eco1/Ctfg7,[4,5] and Trf4.[6] In eukaryotic cells, except at the centromere, cohesin dissociates from chromosomes during the early stages of mitosis. Simultaneously, a related complex condensin binds to sister chromatids.[1,2] A 13S condensin was first identified from *Xenopus* egg extracts by Hirano and coworkers.[7] Condensin consists of Smc2, Smc4, XCAP-D2, XCAP-G, and XCAP-H/Barren.[7] In *S. cerevisiae*, mutants lacking Ycs4, a yeast homolog of XCAP-D2, fail to separate sister chromatids properly during anaphase.[8] However, in *Drosophila* and *C. elegans*, inactivation of condensin disrupts sister chromatid resolution but has little effect on chromosome condensation along the longitudinal axis of sister chromatids,[9,10] which might imply the presence of mitotic activity complementing the function of condensin.[11]

The implication of topoisomerase II in chromosome segregation was originally suggested from its identification as a major component of chromosomes.[12,13] By employing a temperature-sensitive *top2* mutant in yeast[14-16] or utilizing topoisomerase II-specific inhibitors such as ICRF-193 in mammalian cells,[17-19] it was demonstrated that topoisomerase II is essential for chromosome condensation. In addition, when topoisomerase II function is blocked after chromosome condensation, the cells are arrested at metaphase and the chromatids fail to separate.[15,20,21] A functional interplay between topoisomerase II and condensin on mitotic chromosomes has been suggested by the colocalization of topoisomerase II and Barren, a condensin subunit, and their coimmunoprecipitation and interaction in a yeast two-hybrid assay.[22] Despite these observations, the biochemical basis of how topoisomerase II exerts its function in coordination with condensin during mitosis remains to be found.

In this report, we describe a 1.8 MDa multiprotein complex containing topoisomerase IIα. Since topoisomerase IIα is a key regulator of mitotic chromosome structure and topology, we name this newly identified complex the toposome. Seven protein subunits are confirmed, which include topoisomerase IIα, PRP8, RHA, SRPK1, SSRP1, RHII/Gu, and hnRNP C. Employing in vitro assays using chromatinized kinetoplast DNA (k-DNA), we show that the decatenation activity, a key function of topoisomerase IIα to segregate sister chromatids during mitosis, is highly stimulated in the toposome. These observations might provide new insights into the mechanisms of chromosome condensation and segregation.

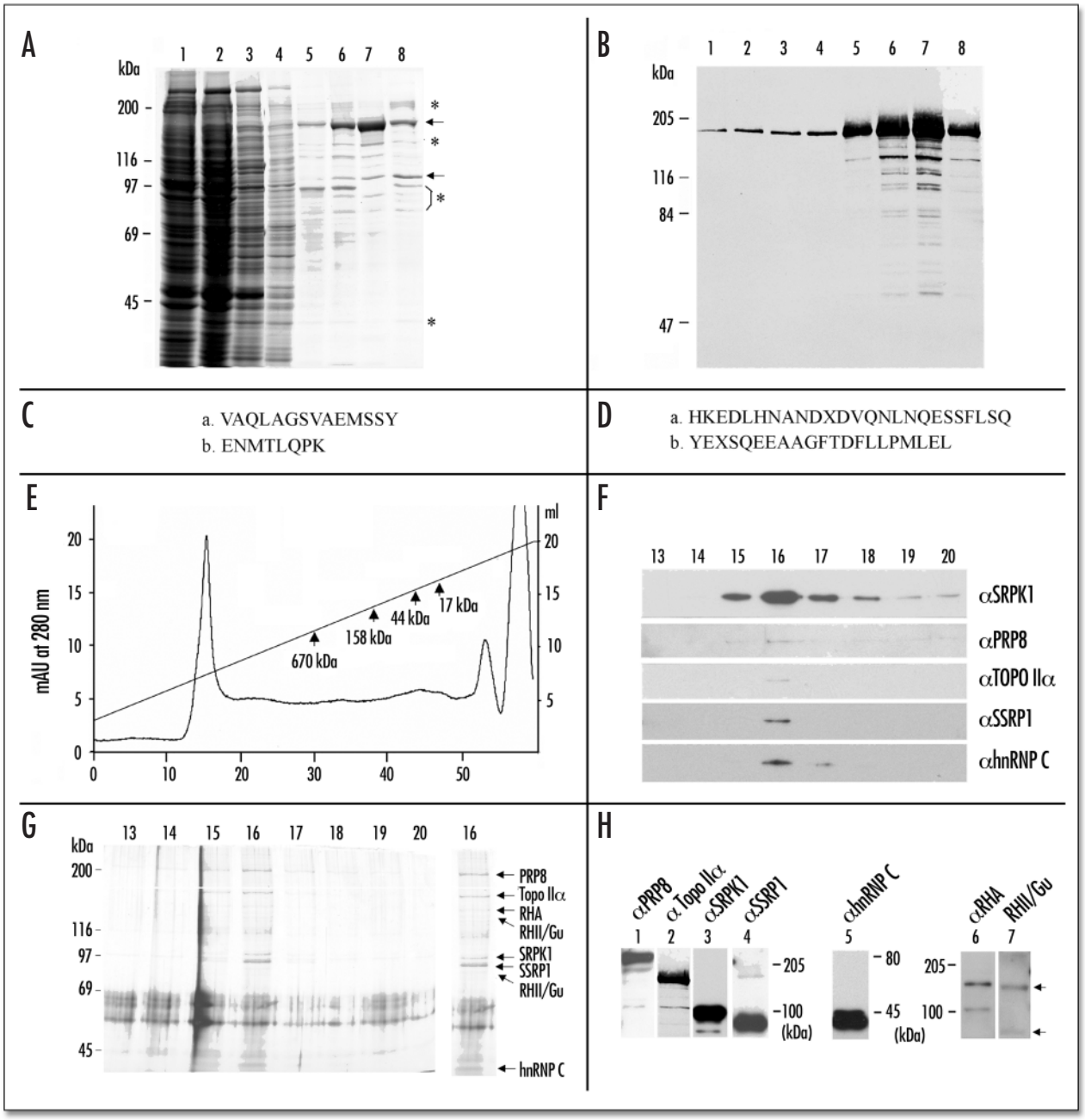

Figure 1. Identification of a multisubunit complex containing topoisomerase IIα and SRPK1. (A) Protein composition of pooled SR kinase fractions, resolved by 7.5 % SDS-PAGE. 1, 50 μg of nuclear extracts; 2, 50 μg of AS 35/65%; 3, 25 μg of DEAE-Sepharose pool; 4, 10 μg of heparin-Sepharose pool; 5, 2.5 μg of phosphocellulose pool; 6, 2 μg of hydroxylapatite pool; 7, 2 μg of green agarose pool I; 8, 1 μg of green agarose pool II. (B) Western blot analysis performed with rabbit polyclonal anti-topoisomerase IIα antibodies. Each lane contained the sample described in panel A but with the following amounts. Lanes 1-4 and 5-8 contained 1 μg and 1.3 μg protein, respectively. (C) & (D) Peptide sequences for 170 kDa (C) and 100 kDa proteins (D), exhibiting perfect matches to human topoisomerase IIα (P11388) and SRPK1 (NP_003128), respectively. (E) Distribution of proteins enriched in pool II in Superose 6 HR column, measured by UV absorbance at 280 nm. (G) Aliquots (30 μl) of indicated fractions were analyzed by 7.5 % SDS-PAGE, followed by visualization using the Silver-Quest silver staining kit (Invitrogen). A protein gel, prepared as in (G), was subjected to Western blot analysis using the indicated antibodies (F). (H) Aliquots (340 ng) of pool II were analyzed by Western blotting with the indicated antibodies.

RESULTS

Copurification of Topoisomerase IIα With SRPK1. Though exhibiting poor similarity, SRPK and Clk/Sky are two families of protein kinases, that phosphorylate SR proteins involved in pre-mRNA splicing.[23,24] In this study, we tested whether there are yet unidentified human nuclear kinases which are specific for nuclear SR proteins. For this purpose, an in vitro kinase assay was employed using a synthetic RS15 peptide (R-S-R-S-R-S-R-S-R-S-R-S-R-S-R-S-R) as substrate, and the SR kinase activity was extensively purified from nuclear extracts of HeLa cells. The presence of β-glycerophosphate in all buffers was essential to prevent inactivation of the SR kinase activity during purification, as seen with ribosomal S6 kinase.[25] After seven successive chromatography steps, the SR kinase activity was divided in two fractions (pools I and II) on a green agarose column. Further analysis revealed that pools I and II contain an identical kinase but different interacting factors (see below). The specific kinase activities of pools I and II were estimated to be approximately 12 and 31 units/μg of protein, respectively.

To identify protein(s) responsible for the SR kinase activity, pooled

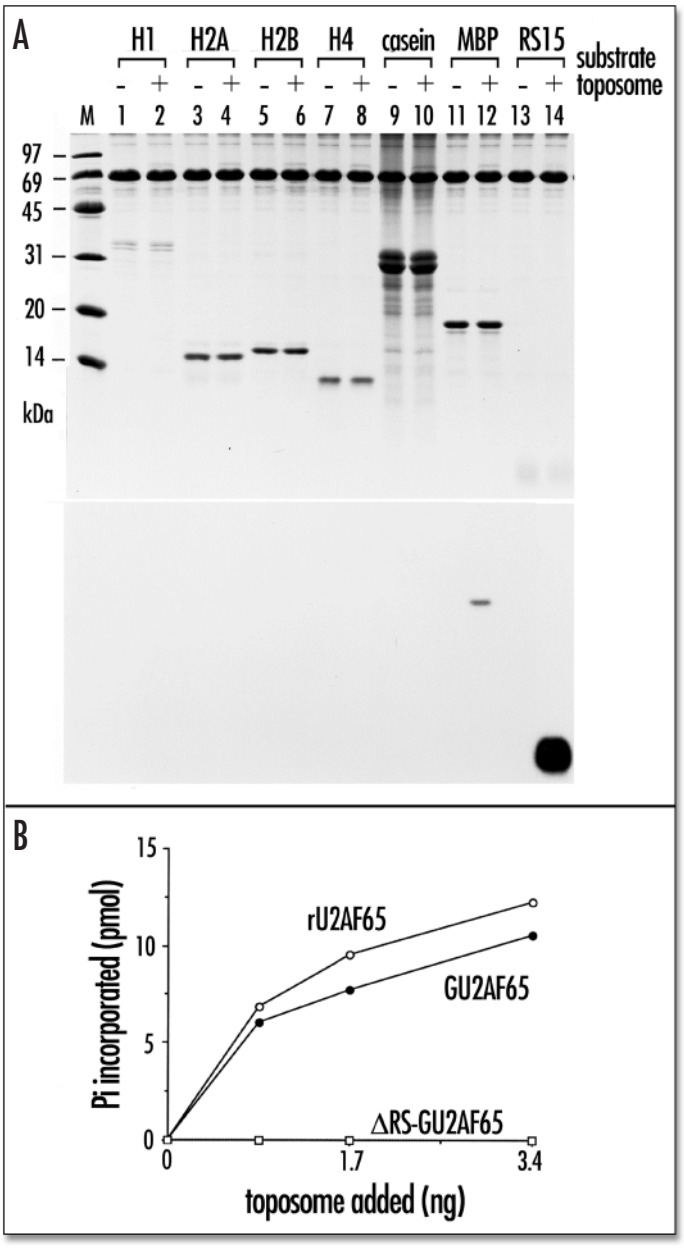

Figure 2. Characterization of the SR kinase activity of toposome. (A) 1 μg of indicated proteins was compared in the standard phosphorylation reaction containing 3.4 ng of toposome. Following electrophoresis on 15% SDS-PAGE, proteins were visualized by coomassie blue staining (upper panel), and phosphorylated proteins were detected by autoradiography and quantified using a Phospholmager (Molecular Dynamics) (lower panel). (B) Recombinant U2AF65 proteins were incubated in the standard phosphorylation reaction containing varying amounts (0.85-3.4 ng) of toposome. Reaction products were resolved by 10% SDS-PAGE, and the extent of phosphorylation was determined as described in (A).

kinase fractions obtained after each column step were resolved by 7.5 % SDS-PAGE. As shown in Figure 1A, a 170 and 100 kDa proteins were enriched in both pools along with the purification of SR kinase. Besides the two proteins, pool II contained several proteins (marked as asterisks in lane 8), including 210, 150, 140, 95, 80, and 43 kDa proteins. The two most distinct 170 kDa and 100 kDa proteins were digested with trypsin and separated by HPLC on a C18 column. Subsequently, microsequencing was performed with two peptides for each protein. For the 170 kDa protein, 8 and 14 residue peptide sequences were obtained (Fig. 1C), and 21 and 25 residue peptide sequences were for the 100 kDa protein (Fig. 1D). Sequence alignment using the Blastp program revealed perfect matches of peptide sequences derived from the 170 kDa and 100 kDa proteins to topoisomerase IIα (P11388) and SRPK1 (NP_003128), respectively.

Identification of a Multisubunit Complex Containing SRPK1 and Topoisomerase IIα. Enrichment of topoisomerase IIα in pool II along with the purification of SRPK1 was confirmed by Western blot analysis (Fig. 1B). To test if topoisomerase IIα and SRPK1 interact with each other, we performed immunoprecipitation (IP) using pool II and antibodies specific for topoisomerase IIα, which showed that SRPK1 is indeed coimmunoprecipitated with topoisomerase IIα (our unpublished data). To further confirm their physical association, pool II was loaded onto a Superose 6 HR column, and the elution profile of SRPK1 and topoisomerase IIα were examined. Unexpectedly, this study yielded only one major protein peak, mainly ranging from fraction 15 to 17 (Fig. 1E) and analysis by 7.5 % SDS-PAGE (Fig. 1F) showed that all identifiable proteins present in pool II coeluted with the peak intensity in fraction 16. This result supports the physical association of topoisomerase IIα with SRPK1 and suggests the presence of a multisubunit complex containing at least 5 proteins in addition to topoisomerase IIα and SRPK1.

Prompted by the above observations, proteins in pool II were subjected to mass spectrometry analysis, which confirmed the identity of SRPK1 and topoisomerase IIα, and also revealed the identity of 210, 150, 140, 95, 80, and 43 kDa proteins as PRP8, RNA helicase A (RHA), RHII/Gu, SSRP1, RHII/Gu, and hnRNP C, respectively. This result was further verified by Western blot analysis employing antibodies specific for the newly identified proteins (Fig. 1H). Relatively weak Western signals for RHA and RHII/GU might suggest their unstable association with the complex. Superose 6HR fractions were also reexamined for the distribution of newly identified proteins employing Western blot analysis. All five major proteins, namely PRP8, topoisomerase IIα, SRPK1, SSRP1, and hnRNP C, yielded positive signals with peak intensity in fraction 16 (Fig. 1G). Since topoisomerase IIα is a key regulator of mitotic chromosome structure and topology, we name this newly identified complex the "toposome". Domain structure of toposome-associated proteins is summarized in Figure 6. Taking the two minor proteins, RHA and RHII/Gu, into account, the molecular weight of toposome is estimated to be about 853 kDa if a dimer of topoisomerase IIα and a single molecule of 6 other proteins constitute the complex. This theoretical value is significantly different from the observed 1.8 MDa (Fig. 1E). It is presently unknown whether such difference is due to an oligomeric state of certain proteins in toposome or toposome itself, or due to unusual conformation of toposome.

Substrate Specificity of the SRPK1 Complexed in Toposome. To determine substrate specificity of SRPK1 complexed in toposome, histone proteins, casein, and myelin basic protein were compared in the standard phosphorylation reaction containing toposome. Among various substrates tested, only MBP and RS15 peptide were phosphorylated (Fig. 2A, lower panel). It was estimated that 3.4 ng of toposome (about 1.9 fmol with assumed molecular weight of 1.8 MDa) incorporated about 3 and 40 pmol of phosphates into MBP and RS15 peptides, respectively. These results indicate that the SRPK1 in toposome is highly active and retains substrate specificity. To further verify the substrate specificity of SRPK1 in toposome, rU2AF65, GU2AF65, and a mutant (ΔRS-GU2AF65) lacking the RS dipeptide[26] were compared in the phosphorylation reaction containing increasing amounts of toposome. As shown in Figure 2B, rU2AF65 (open circle) and GU2AF65 (closed circle) were phosphorylated by toposome with almost comparable efficiency. In contrast, phosphorylation of ΔRS-GU2AF65 (open square) was not detected under the conditions used. This result demonstrates that the association with toposome does not alter the substrate specificity of SRPK1.

Decatenation Activity of Topoisomerase IIα Complexed in Toposome. Next, we determined whether topoisomerase IIα complexed in toposome is as active as free topoisomerase IIα. Since it contains 3.7-fold higher topoisomerase IIα but 2.5-fold less SRPK1 compared to pool II, and lacks other components of toposome, pool I was used as a source of free topoisomerase IIα in the present study. As shown in Figure 3A, 1.9 ng of topoisomerase IIα from pool I mediated a complete decatenation of k-DNA (0.3 μg) (Fig. 3, lane 3). Almost comparable or slightly inefficient decatenation of k-DNA was

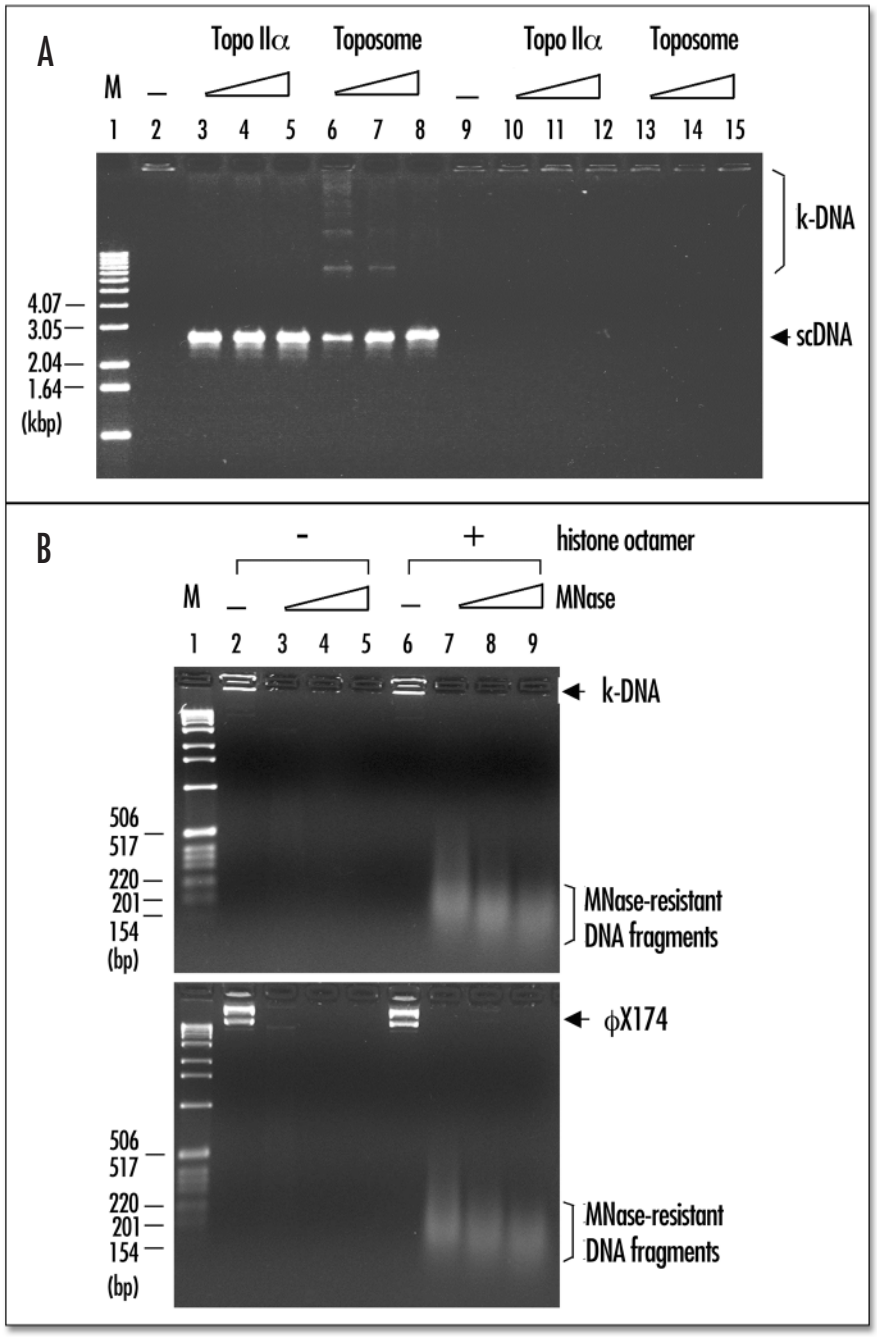

Figure 3. In vitro analysis of decatenation reactions mediated by topoisomerase IIα and toposome. (A) Decatenation activity was measured in a reaction mixture containing increasing amounts of topoisomerase IIα (1.9–7.6 ng) (lanes 3–5 and lanes 10–12) or toposome (2.1–8.4 ng) (lanes 6–8 and lanes13–15) and 0.3 μg of either histone-free (lanes 2–8) or chromatinized k-DNA (lanes 9–15). Following incubation for 30 min at 37°C, reaction mixture was analyzed on 1% agarose gel. Supercoiled circular DNA (scDNA) and catenated DNAs (bracket) are indicated. (B) *Tetrahymena* kinetoplast DNA (k-DNA) (upper panel) or ϕX174 form I DNA (lower panel) was chromatinized, as described in "Experimental Procedures". One μg of histone-free DNA (k-DNA or ϕX174) or its chromatinized form was incubated with 0.1, 1, or 10 units of micrococcal nuclease (Worthington), and analyzed on 1.5% agarose gel. Parental DNAs and their derivatives resistant to micrococcal nuclease are indicated.

obtained with 8.5 ng of toposome equivalent to 2.2 ng of topoisomerase IIα (Fig. 3, lane 8). Thus, it appears that the association of topoisomerase IIα with toposome does not significantly alter the decatenation activity.

For efficient decatenation of sister chromatids during mitosis, topoisomerase IIα should be able to access sister chromatids undergoing progressive condensation. The presence of SSRP1, a HMG protein,[27] led us to explore whether topoisomerase IIα complexed in toposome gains better access to chromatin. To test this possibility, we chromatinized k-DNA using HeLa core histones, and compared toposome and topoisomerase IIα for their ability to decatenate chromatinized k-DNA. The result that k-DNA became resistant to micrococcal nuclease once chromatized with core histones, generating nuclease-resistant DNA fragments of approximately 150 bp, similar to the minimal length of nucleosomal DNA,[28] indicates that although irregularly spaced,[29] nucleosomes were efficiently formed on k-DNA (Fig. 3B, upper panel). Next, we compared topoisomerase IIα and toposome for their ability to decatenate chromatinized k-DNA. As described in Figure 3A, both topoisomerase IIα and toposome efficiently monomerized 0.3 μg of histone-free k-DNA (Fig. 3, lanes 3 and 8). It was estimated that approximately 5.6 fmol of topoisomerase IIα dimer catalyzed the decatenation of about 150 fmol of k-DNA. Under the same reaction condition, however, there was no noticeable decatenation of chromatinized k-DNA (Fig. 3, lanes 10–15).

Topoisomerase IIα is an abundant nuclear protein, present every 25 kb of chromosomal DNA in HeLa cells, and the majority of topoisomerase IIα associates with chromosomes during mitosis.[12,13,30] Therefore, we speculated that the decatenation of chromatin DNA would be efficient if topoisomerase IIα is provided in stoichiometric amounts relative to chromatin substrate. Indeed, chromatinized k-DNA was efficiently decatenated by higher levels of topoisomerase IIα or toposome in an ATP-dependent manner (Fig. 4A, compare lanes 3–6 with lanes 7–10). It was estimated that 135 ng of topoisomerase IIα in toposome decatenated about 75% of input chromatinized k-DNA, whereas the comparable amount of free topoisomerase IIα decatenated only 14% of input chromatinized k-DNA (Fig. 4B).

Knotted Circular DNAs Were Generated from Catenated DNA by Topoisomerase IIα. Reaction products obtained with chromatinized k-DNA appear to be more complex in DNA composition than those with histone-free k-DNA (compare Fig. 3A, lane 6 with Fig. 4A, lane 6). Among them, most intriguing was the one migrating faster than supercoiled circular DNA. To identify the conformational state of decatenated DNA products, reaction mixture was subjected to 2-D gel electrophoresis, which revealed that decatenated DNA products could be classified into three different conformers, including highly packed knotted DNA (kc), supercoiled DNA (sc), and nicked circular DNA (oc) (Fig. 4E). For illustrative purpose, they are grouped by line according to the conformation state (Fig. 4F).

Once provided in 1–3 molar ratio relative to DNA substrate, the budding yeast topoisomerase II efficiently knots any topologically closed DNA template, either relaxed or supercoiled DNA.[31,32] Since decatenation reaction in the present study was performed under similar conditions, it is possible that circular monomers, either relaxed or supercoiled, are generated first in the decatenation reaction, and subsequently further converted into knotted form by toposome. If this is the case, it is predicted that knotted DNA would be more efficiently generated in a reaction provided with circular DNA monomer. To test this possibility, ϕX174 DNA was chromatinized using HeLa core histones (Fig. 3B, lower panel), and chromatinized ϕX174 DNA was incubated with increasing amounts of toposome or topoisomerase

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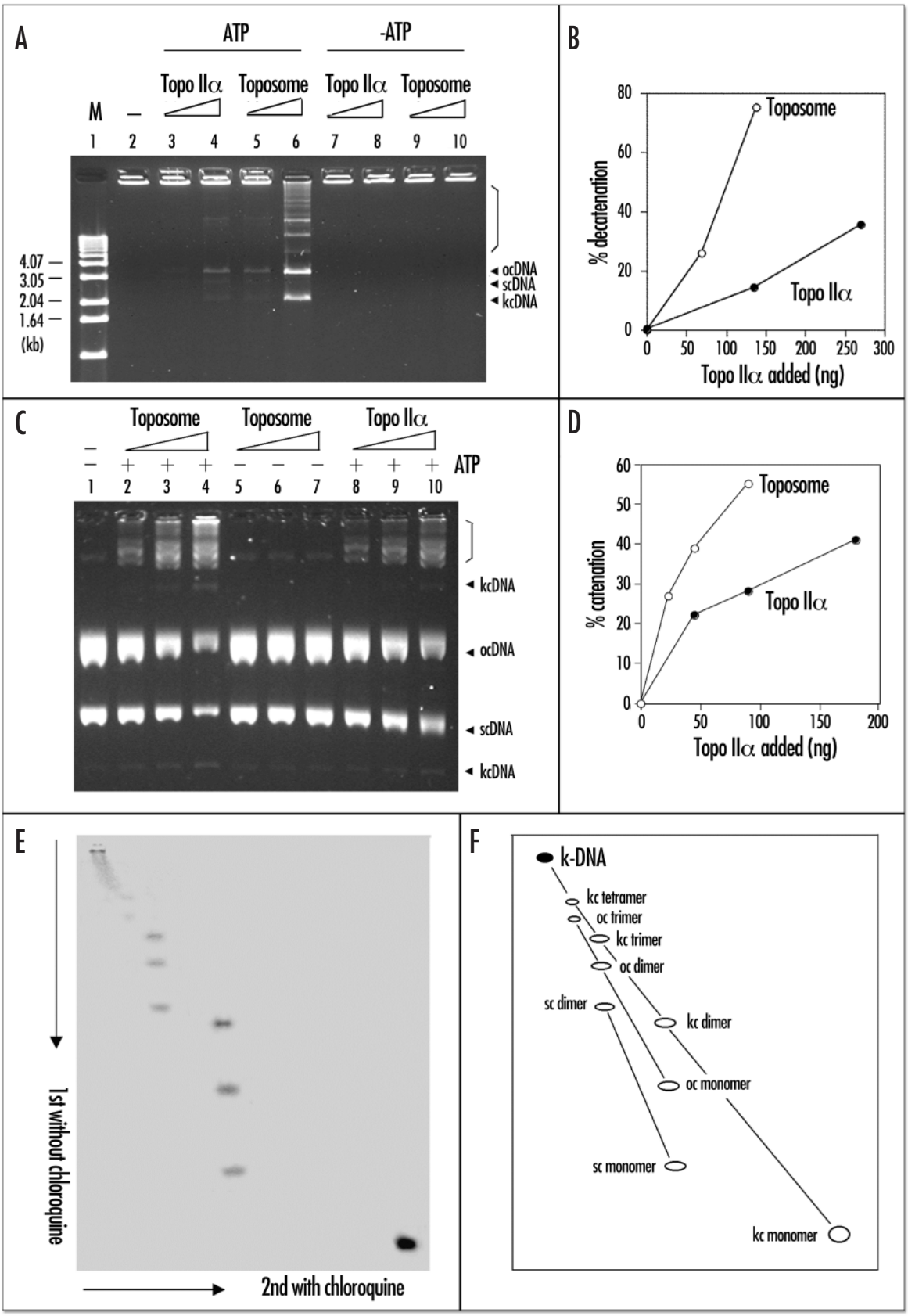

IIα. As shown in Figure 4C, catenated DNA oligomers (bracket) were generated from both supercoiled (scDNA) and relaxed circles (ocDNA) by the ATP-dependent function of toposome (compare lanes 2-3 and 5-7) or topoisomerase IIα (lanes 8–10). In contrast to the above prediction, however, knotted DNA was poorly produced. These results suggest that knotting of circular DNA is mechanistically coupled to the decatenation reaction catalyzed by topoisomearse IIα.

Dynamic Nature of Toposome and Decatenation Activity During the Cell Cycle. To determine the subcellular localization of toposome-associated proteins throughout the cell cycle, both cytoplasmic and nuclear extracts were prepared from HeLa cells synchronized in G_1/S, S, or G_2/M phase. As shown in Figure 5A, the presence of topoisomerase IIα in the nucleus

Figure 4 (Left). In vitro decatenation and catenation of chromatinized DNAs. (A and B) Decatenation activity was measured with increasing amounts of topoisomerase IIα (135-270 ng) (lanes 3–4 and 7–8) or toposome containing 68-135 ng of topoisomerase II (lanes 5–6 and 9–10) and 0.3 μg of chromatinized k-DNA in the presence of ATP (lanes 3-6) or in its absence (lanes 7-10). Relaxed open circular DNA (ocDNA), supercoiled circular DNA (scDNA), knotted circular DNA (kcDNA), and catenated DNAs undergoing decatenation (bracket) were quantified and compared to catenated DNA retained on the well using the QuantityOne software, which is presented as percent decatenation in panel B. (C and D) Catenation activity was measured with amounts of topoisomerase IIα (45-180 ng) (lanes 8–10) or toposome containing 23-90 ng of topoisomerase IIα (lanes 2–7) and 0.3 μg of chromatinized ϕX174 DNA in the presence of ATP (lanes 2–4 and 8–10) or in its absence (lanes 5–7). Catenated DNAs (bracket) and knotted circular DNA (kcDNA) were quantified and compared to the remaining relaxed open circular DNA (ocDNA) and supercoiled circular DNA (scDNA), which is presented as percent catenation in panel D. (E and F) Reaction products, obtained as described in Figure 4A, lane 6, were analyzed by 2-D gel electrophoresis. For illustrative purposes, original picture is also shown in scheme (F), which indicates the relative position of three DNA conformers such as supercoiled circular form (sc monomer and dimer), open circular form (oc monomer to trimer), and knotted circular form (kc monomer to tetramer). The catenated k-DNA is depicted as closed circular oval.

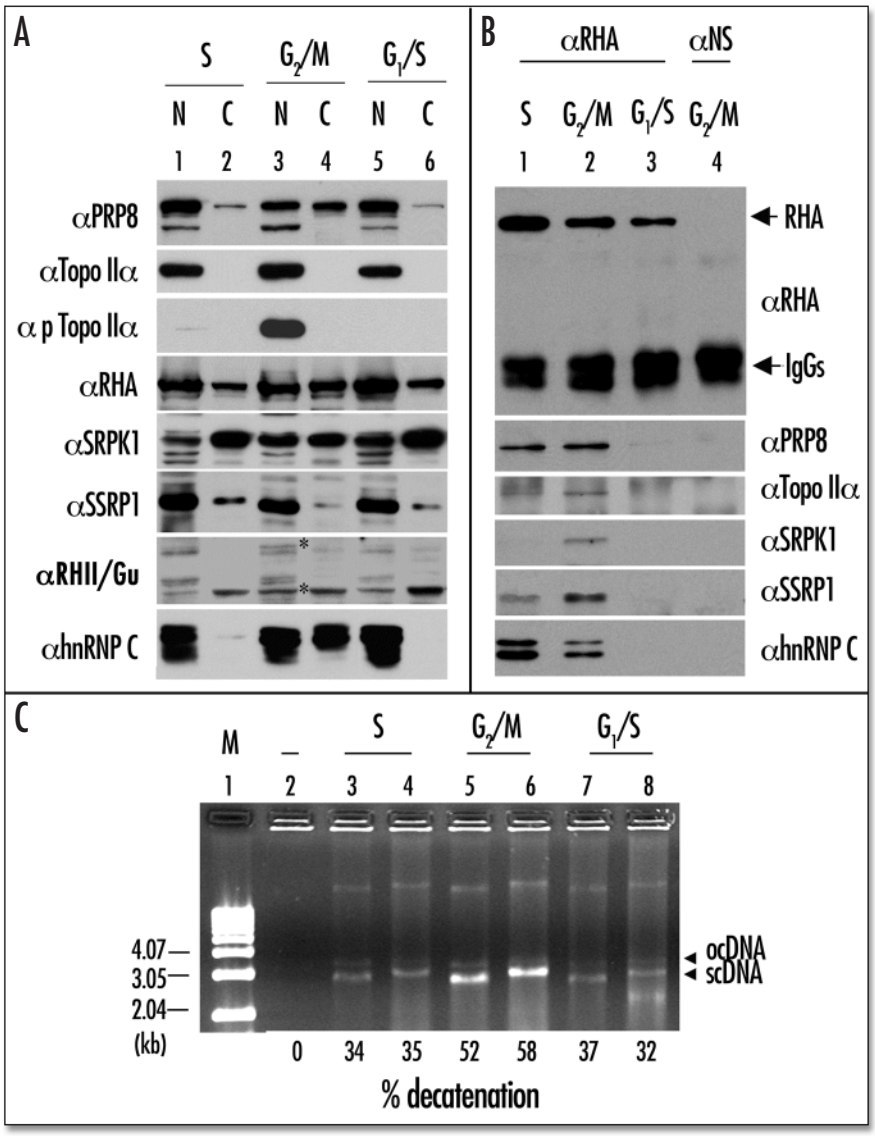

Figure 5 (Above). Cell cycle-dependent subcellular localization of proteins associated with toposome and nuclear decatenation activity. (A) Cytoplasmic and nuclear extracts, prepared at indicated phases of the cell cycle, was examined for the presence of toposome-associated proteins employing Western blot analysis. (B) Immunoprecipitation was performed with aliquots (600 μg) of nuclear extracts and 10 μg of either polyclonal anti-RHA antibodies (α RHA) or preimmune antibodies (α NS). The IP pellets were subjected to Western blot analysis for the presence of toposome-associated proteins. (C) Ten μg (lanes 3, 5 and 7) or 20 μg (lanes 4, 6 and 8) of nuclear extracts were examined for decatenation activity using 0.3 μg of chromatinized k-DNA. Following 1 hr incubation at 37°C, reaction mixture was analyzed on 1% agarose gel. Open circular DNA (ocDNA) and supercoiled circular DNA (scDNA) are indicated.

throughout the cell cycle was detected with polyclonal anti-topoisomerase IIα antibodies (α Topo IIα). Furthermore, Western blot analysis using anti-P1212 antibodies (α pTopo IIα) specific for a phosphorylated form of topoisomerase IIα at amino acid position 1212, formed during mitosis,[18] revealed the presence of phosphorylated topoisomerase IIα in the nuclear extracts of G_2/M cells. These results indicate that HeLa cells were appropriately synchronized at specific stages of the cell cycle. Subsequently, extracts were examined for the presence of proteins associated with toposome. SSRP1 was detected mainly in the nuclear fraction throughout the cell cycle. PRP8 and hnRNP C were predominantly detected in nuclear extracts derived from interphase cells, but during the G_2/M phase, significant fractions were relocalized into the cytoplasm. Though less severe, RHA showed a similar pattern. However, opposite results were obtained with SRPK1 and RHII/Gu. Although higher levels of SRPK1 were detected in the cytoplasmic fraction during interphase, there was a noticeable increase in nuclear SRPK1 during the G_2/M phase. In the case of RHII/Gu, Western blotting yielded multiple signals in addition to the two (asterisks) seen with toposome (Fig. 1H), which might be due to covalent modification, isoforms, or cross-reactivity of antibodies. Taken together, these results indicate that although subcellular localization dynamically changes throughout the cell cycle, all toposome-associated proteins are present in the nucleus during mitosis.

Previously we demonstrated the interaction between RHA and topoisomerase IIα using anti-RHA antibodies.[33] Employing essentially the same immunoprecipitation (IP) method, we examined how interaction among toposome-associated proteins changes throughout the cell cycle. As shown in Figure 5B, RHA was detected in the IP pellets obtained with immune antibodies specific for RHA (α RHA) (Fig. 5, lanes 1–3) but not with its preimmune antibodies (α NS) (Fig. 5, lane 4). All toposome-associated proteins were detected in the IP pellets obtained from nuclear extracts of G_2/M phase cells (Fig. 5, lane 2) but were not detected in the IP pellets of G_1/S phase cells (Fig. 5, lane 3) nor in the IP pellets of preimmune antibodies (Fig. 5, lane 4). In addition, relatively poor immunoprecipitation of RHA was observed from nuclear extracts of G_1/S phase cells (Fig. 5, lane 3). These results might suggest a more complex identity of RHA in G_1/S phase that interferes with the access of antibodies. Alternatively, RHA interaction with toposome- associated proteins might occur specifically in G_2/M phase. Although comparative analysis of RHA-containing complexes at different stages of the cell cycle is necessary to address the above possibilities, our results indicate that toposome is a dynamic complex whose assembly or activation is subject to cell cycle regulation.

We also explored the possibility that decatenation activity may be modulated in a cell cycle-dependent manner. For this purpose, decatenation activity

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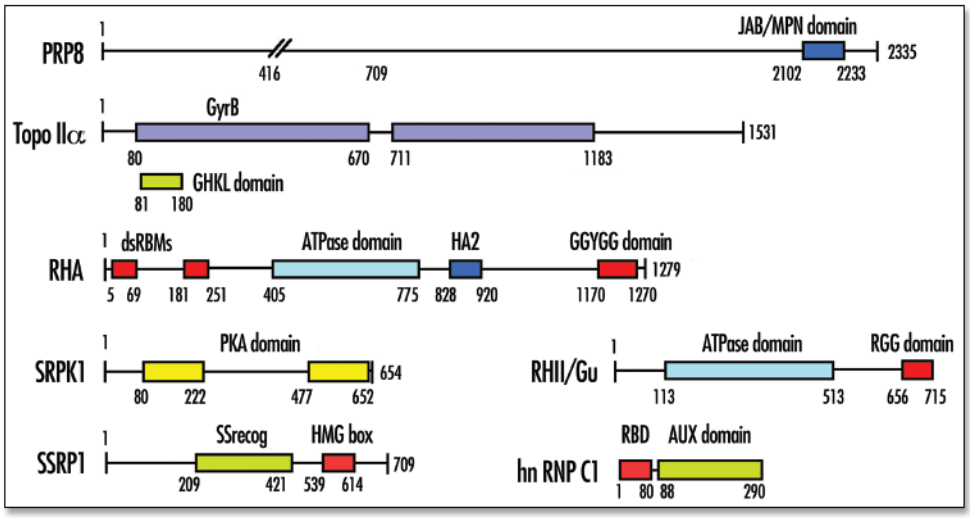

Figure 6. Schematic representation of the domain structure of proteins associated with toposome. Amino acid residues and domain structures are from the NCBI genbank accession number NP_006436 for PRP8, P11388 for topoisomerase IIα, NP_001348 for RHA, NP_003128 for SRPK1, NP_003137.1 for SSRP1, NP_004719 for RHII/Gu, and NP_004491 for hnRNP C1.

was measured in nuclear extracts prepared form HeLa cells synchronized at the indicated cell cycle in reaction mixtures containing chromatinized k-DNA. It should be noted that nuclear extract contains not only topoisomerase IIα but also topoisomerase IIβ. It is also possible that only a fraction of nuclear topoisomerase IIα is engaged in the formation of toposome. Nonetheless, significant decatenation activity was detected with nuclear extract of the G_2/M phase cells (Fig. 5C). Since higher levels of protein did not significantly increase the decatenation of chromatin substrate (compare Fig. 5, lanes 3, 5 and 7 with Fig. 5, lanes 4, 6 and 8), the decatenation activity observed only with lower levels of protein was compared among the different extracts. Consistent with interaction among toposome-associated proteins, this result indicates that the decatenation activity, essential for the separation of sister chromatids, is subjected to cell cycle regulation. Further studies are underway to establish whether the assembly of toposome is responsible for the enhanced decatenation activity during mitosis, which could provide new insights into the regulation of the DNA topology during the cell cycle, especially, from the aspect of chromosome condensation and separation (see "Discussion").

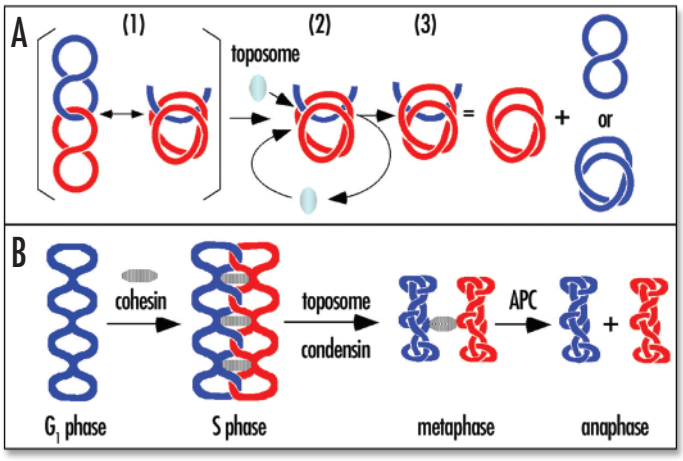

Figure 7. A hypothetical model for the decatenation coupled with knotting of circular DNAs (A) and of mitotic chromosomes (B).

DISCUSSION

In this report, we describe the isolation and identification of a multisubunit complex. This newly discovered complex, named "toposome", was estimated to be as large as 1.8 MDa. Both SRPK1 and topoisomerase IIα complexed in toposome were highly active in reactions for SR domain-specific phosphorylation and decatenation of catenated DNAs, respectively. During their purification, it was observed that toposome was retained on a green agarose column and eluted by 0.4 M KCl, whereas the majority of topoisomerase IIα and SRPK1 were recovered in the flow-through fraction containing 0.2 M KCl. These results suggest that although topoisomerase IIα stably interacts with SRPK1, not all of them are engaged in the formation of toposome. It is presently unknown whether their interaction reflects an intermediate step for the assembly of toposome or its disintegration.

The domain structure of seven factors constituting toposome is depicted in Figure 6. When measured with nuclear extracts, the highest decatenation activity was observed at the G_2/M phase. The present study also shows that interaction of RHA with topoisomerase IIα and other toposome subunits is highly stimulated at the G_2/M phase. Thus, it is tempting to propose that toposome is assembled or activated in a cell cycle-specific manner to facilitate the segregation of sister chromatids. What would be the underlying mechanism of assembly or activation of toposome in G_2/M phase? The Dsk1, a SRPK1 homolog in *S. pombe*, is exclusively present in the cytoplasm in the interphase cells but is localized to the nucleus during mitosis.[34] In contrast, SRPK1 in humans is present in both cytoplasm and nucleus.[35] Despite this difference, SRPK1 is most active in mitosis,[23] and increased nuclear localization of SRPK1 has been observed in G_2/M phase in the present study. These results suggest that the activation of SRPK1 might serve as a key step for the assembly of toposome.

Once constituted in toposome, topoisomerase IIα becomes highly reactive with chromatin DNA. Two models could explain the activation of topoisomearse IIα in toposome. First, topoisomerase IIα might be modified during toposome assembly, which, in turn enhances its catalytic activity. Although it has been known that topoisomerase IIα is phosphorylated by a variety of protein kinases, including CKII,[36,37] MAP kinase,[38] and protein kinase C,[39,40] we were unable to find any change in decatenation activity due to phosphorylation (our unpublished data). Considering that SSRP1 forms a heterodimeric complex FACT with human Cdc68/Spt16 that facilitates transcription elongation on the chromatin template,[41,42] we are now exploring an alternative possibility that enhanced decatenation might be due to better access of toposome to chromatin DNA.

It should be noted that at least three proteins constituting toposome are functionally linked to pre-mRNA splicing, which include PRP8,[43] SRPK1,[23] and hnRNP C.[44] Among them, implication of PRP8[45] and SRPK1[34] in cell cycle progression has been interpreted as a requirement for the splicing of pre-mRNAs of key cell cycle regulators.[46] Another model envisions that some splicing factors such as SRPK1, tightly associated with nuclear matrix,[47] facilitate

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disruption of the spliceosome as a prerequisite for nuclear division.[46] Identification of toposome provides an alternative function of PRP8 and SRPK1. A number of noncoding RNA are involved in the regulation of gene expression.[48] *XIST/Xist* in mammals, for example, functions as a key regulator for X chromosome inactivation.[49,50] However, it is presently unclear whether they remain associated with chromosome during mitosis or whether they are excluded from condensing chromosome.[51,52] Whatever the underlying mechanisms could be, RNA-interacting activity should be required either for the removal of chromatin-associated RNA or for the orderly segregation of sister chromatids containing RNA. Since toposome contains at least four proteins harboring RNA-interacting activity such PRP8, RHA, RHII/Gu, hnRNP C, perhaps it plays a central role in the regulation of chromatin loaded with RNA during mitosis.

It has been proposed that the introduction of global positive writhes into DNA by condensin serve as potential mechanisms of DNA compaction in mitosis.[53] Since the above observation was obtained using relaxed circular DNA, there still exists a large gap in our understanding of actual folding of mostly negatively supercoiled chromatin DNA within a native chromosome during mitosis. In this regard, our results, obtained in vitro using chromatinized DNAs, might provide another possibility for the mechanism of chromosome condensation during mitosis.

The present study show that topoisomerase IIα, either free or complexed within toposome, mediates the decatenation of chromatinized catenated k-DNA at the expense of ATP, which produces three different DNA conformers such as closed, relaxed, and knotted circular DNA. The inability of topoisomerase IIα to efficiently generate knotted DNA from monomeric DNA, of either relaxed or closed circular conformation, indicates that knots are generated from the catenated DNA. These observations raise an intriguing possibility that the decatenation and knotting of chromatin DNA might be mechanistically coupled, as depicted in Figure 7A. The mechanism of action of topoisomerase IIα in this model, mediating the strand passage reaction of intramolecular DNA crossover, is fundamentally the same as previously proposed by Kimura et al. (1999). In brief, two negatively supercoiled circular DNAs are entangled with each other. In step 1, the bottom circle of DNA (red) flips, placing itself in close proximity to the top circle. Toposome recognizes both inter- and intramolecular DNA crossovers (arrows, step 2), and catalyzes strand passage reactions (step 3). The strand passage reaction involving intermolecular DNA crossover results in the separation of two circular DNAs, whereas the one with intramolecular DNA crossover yields a knotted circular DNA.

It is believed that decatenation of the two entangled sister chromatid DNAs is functionally coupled with condensin-mediated compaction during prometaphase.[7,54-57] It is intriguing to compare our proposed model for decatenation coupled with knotting to structural changes of the two sister chromatids. In Figure 7B, two sister chromatid DNAs, depicted in blue and red, are held together by cohesin complex (hatched gray bar). The concerted function of toposome and condensin mediates the simultaneous separation and condensation of two sister chromatids. Determination of the impact of condensin on the decatenation of chromatized DNA catalyzed by toposome, and if condensin directly interacts with toposome, should provide more insights into how condensin and topoisomerase IIα coordinate their function during mitosis. Studies are underway to address how decatenation activity of topoisomerase IIα is activated in toposome and whether assembly and/or activation of toposome are subject to cell cycle regulation.

MATERIALS AND METHODS

Purification of Toposome. All purification steps were carried out at 4°C, and protein was measured using Bradford reagent (Bio-Rad) with albumin as standard. Nuclear extracts (2.3 g), prepared from HeLa cells (60 liters), was subjected to ammonium sulfate (AS) fractionation from 0-35% saturation to 35–65% and 65–95% saturation. The 35-65% AS fraction (1.1 g protein) was dialyzed overnight against buffer B (20 mM Hepes-NaOH, pH 7.4, 10 mM β-glycerophosphate, 0.1 mM EDTA, 2 mM DTT) containing 0.1 M-KCl and 20 % glycerol (4 liters), and loaded onto a DEAE-Sepharose column (2.5 x 10 cm) equilibrated with buffer B containing 0.1 M KCl and 10 % glycerol. The flow-through fraction (0.94 g) was loaded onto a heparin-Sepharose column (2.5 x 10 cm). Again, the flow-through fraction (606 mg) was loaded onto a phosphocellulose column (2.5 x 10 cm), and the protein adsorbed to the column was eluted with a linear gradient (400 ml) of KCl (0.1–1 M) in buffer B containing 10 % glycerol. The SR kinase activity, detected in fractions containing about 0.75 M KCl, was pooled (36 mg) and dialyzed overnight against 4 liters of buffer B containing 0.3 M KCl and 20% glycerol. Subsequently, they were loaded onto a brown agarose column (1.5 x 10 cm). The flow-through and 0.5 M-KCl fractions containing SR kinase activity were combined (11 mg) and directly loaded onto a hydroxyapatite column (1.5 x 5 cm) equilibrated with buffer B containing 20 mM potassium phosphate, pH 7.4, 0.1 M KCl, and 10 % glycerol. The bound protein was eluted with a linear gradient (60 ml) of potassium phosphate (20 mM-0.7 M), pH 7.4, in buffer B containing 0.1 M KCl and 10 % glycerol. The SR kinase activity, detected in fractions containing about 0.3 M potassium phosphate, was pooled and dialyzed overnight against 2 liters of buffer B containing 0.2 M KCl and 20% glycerol, and then loaded onto a green agarose column (1 ml). After washing the column with 2 ml of buffer B containing 0.2 M KCl and 10% glycerol, bound proteins were eluted with a linear gradient (20 ml) of KCl (0.3–1 M) in buffer B containing 10% glycerol. The SR kinase activity was detected in the flow-through (pool I, 840 μg) and 0.4 M KCl-fractions (pool II, 270 μg). Employing the Akta purifier (Amersham-Pharmacia), an aliquot of pool II (34 μg) was loaded on a Superose 6 HR column equilibrated with buffer C containing 20 mM Hepes-NaOH, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 5 mM β-glycerophosphate. A total of 50 fractions were collected at a rate of 0.3 ml per min per fraction using buffer C after discarding the first 3 ml. Molecular weight standard for column calibration was from Bio-Rad.

Cell Synchronization. HeLa cells were seeded to 10 % confluence in 3 groups (A–C) of ten 90 mm culture plates. The following day, cells were replenished with Dulbeco's modified Eagle's media (DME) containing 2 mM thymidine and 7% fetal bovine serum (FBS). After 16 hr-incubation, culture plates were washed twice with serum-free DME, then supplemented with DME containing 24 μM deoxycytidine and 7% FBS. After incubation for 9 hr at 37°C, cells were washed twice with serum-free DME, provided with DME containing 2 mM thymidine and 7% FBS, and then continuously incubated for 20 hr. Cells in group A were harvested immediately and used to prepare cytoplasmic and nuclear extracts. Cells in group B were incubated for an additional 7 hr in DME containing 7% FBS, but those in group C were grown for 18 hr in DME containing 7% FBS and 0.2 μM nocodazole (Sigma). Cytoplasmic and nuclear extracts were prepared from cells in groups A–C, as described previously.[58]

Protein in-gel Digestion and Identification by Mass Spectrometry. Proteins, visualized by coomassie blue staining, were excised and digested overnight at 37°C in 25 mM ammonium bicarbonate (pH 8.0, 20 μl) containing 0.2 μg of modified trypsin (Promega). Peptides were extracted and analyzed by MALDI-TOF mass spectrometry (MS) using 4700 Proteomics Analyzer (Applied Biosystem). MALDI-TOF MS was performed in the delayed extraction and reflector mode using a matrix of α-cyano-4-hydroxy-cinnamic acid. Bradykinin (100 fmol/μl) and ACTH 18-39 (200 fmol/μl) were used as internal standards for calibration. One μl of sample was mixed with 1 μl of matrix and 0.4 μl of internal standard mix, and an aliquot (2 μl) was applied to the sample plate. Each spectrum was accumulated over approximately 2000 laser shots. Measured peptide masses were compared to the NCBI nonredundant (nr) protein sequence database using MS-Fit search programs.

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MASCOT and ProFound were also used to confirm database search results. All searches were performed with a mass tolerance of 0.005% error (50 ppm), and a positive identification required a minimum of four peptide matches and the highest probability ranking in at least two of the search programs employed.

HPLC Fractionation and Peptide Sequencing. For identification by Edman degradation, approximately 10 μg and 5 μg of the 170 kDa and 100 kDa proteins, respectively, were excised from the gel and digested as described above. Subsequently, peptides were separated on a Kingsorb C18 RP column (150 x 4.5 mm) (Phenomenex) using 0–60% acetonitrile gradient in 0.1% TFA (60 ml). Among peptides collected, T35 and T50 obtained from the 100 kDa protein and T13 and T86 from the 170 kDa protein, were dried and loaded on the Procise 494 protein sequencer (Applied Biosystems). Peptide sequences were analyzed using SequencePro® software and were identified by sequence alignment to the NCBI nr protein sequence database.

Protein Kinase Assay. Unless otherwise indicated, the SR kinase activity was measured in a reaction mixture (15 μl) containing 20 mM Hepes-NaOH, pH 7.4, 2 mM DTT, 3 mM $MgCl_2$, 0.2 mM γ-^{32}P-ATP (4400 cpm/pmol), 0.2 mg/ml BSA, 1 μg of RS15 peptide (R-S-R-S-R-S-R-S-R-S-R-S-R-S-R), and the indicated amount of toposome. During the purification of SR kinase, each column fraction was diluted 50-fold in a buffer containing 20 mM Hepes-NaOH, pH 7.4, 2 mM DTT, 50 mM KCl, 0.2 mg/ml BSA, 0.05% NP-40, and 10% glycerol, and aliquots of the diluted fractions (1 μl) were used for the kinase activity assay. Mixtures were incubated for 30 min at 37°C and the reaction was stopped by the addition of either 0.1 ml of 50 mM sodium pyrophosphate, pH 7.0, containing 10 mg/ml BSA for the TCA precipitation or 5 μl of 4x SDS sample buffer for the electrophoretic analysis of the phosphorylated protein. One unit of RS kinase activity was defined as the amount of protein required for the incorporation of 1 nmol of phosphate into the substrate under the conditions described above.

Chromatin Assembly In Vitro. Twenty μg of *Tetrahymena* kinetoplast DNA (k-DNA) (Topogen) or ϕX174 form I DNA was incubated for 30 min at 37°C in a reaction mixture (200 μl) consisting of 50 mM Hepes-NaOH, pH 7.4, 1 M NaCl, 0.5 mM PMSF, and 25 μg of HeLa core histones prepared as described previously.[59] After 30 min-incubation at 37°C, the NaCl concentration was serially lowered by the addition of 35, 72, 93 μl of dilution buffer containing 50 mM Hepes-NaOH, pH 7.4, 1 mM EDTA, and 0.5 mM PMSF. Each dilution was followed by a 20-min incubation at 30°C. After the final dilution step, the reaction mixture was incubated at 4°C overnight, it was then dialyzed for 4 hr against 1 liter of a buffer consisting of 10 mM Tris-HCl, pH 7.4, 0.5 mM PMSF, 10 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 0.05% NP-40, and 10% glycerol. To determine nuclease sensitivity, 1 μg of histone-free DNA (k-DNA or ϕX174) or its chromatinized form was added in a reaction mixture (10 μl) containing 20 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 50 mM NaCl, and the indicated amount of micrococcal nuclease (Worthington) reconstituted in 20 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, and 20% glycerol. After incubation for 5 min at 37°C, reaction mixture was transferred to ice and mixed with EGTA to a final concentration of 2 mM, SDS to 0.5%, and proteinase K (Boehringer Manheim) to 0.5 mg/ml, and then analyzed on 1.5% agarose gel in 0.5x TBE. Intact or partially hydrolyzed DNAs were visualized by staining with ethidium bromide and documented using Gel-Doc system (Bio-Rad).

Decatenation and Catenation Assays. Reaction mixture (10 μl), containing 50 mM Tris-HCl, pH 8.0, 0.1 M KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 3 mM ATP, 0.2 mg/ml BSA, and 0.3 μg of either histone-free or chromatinized DNA, was incubated for 30 min at 37°C. Subsequently, the reaction mixture was supplemented with SDS and proteinase K to the final concentration of 0.5% and 0.5 mg/ml, respectively, incubated for an additional 30 min at 37°C, and analyzed on 1% agarose gel in 0.5x TBE. For 2-D gel electrophoresis, the reaction mixtures containing decatenated products were resolved on 1% agarose gel for 16 hr at 30V in 1x TBE. Subsequently, the gel was soaked in 3 μg/ml chloroquine in 1x TBE for 8 hr, then shifted 90°C from its original position, and reelectrophoresed in the second dimension for 16 hr at 30 V in 1x TBE containing 3 μg/ml chloroquine.

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