Functional interactions of DNA topoisomerases with a human replication origin

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The human DNA replication origin, located in the lamin B2 gene, interacts with the DNA topoisomerases I and II in a cell cycle-modulated manner. The topoisomerases interact *in vivo* and *in vitro* with precise bonds ahead of the start sites of bidirectional replication, within the pre-replicative complex region; topoisomerase I is bound in M, early G1 and G1/S border and topoisomerase II in M and the middle of G1. The Orc2 protein competes for the same sites of the origin bound by either topoisomerase in different moments of the cell cycle; furthermore, it interacts on the DNA with topoisomerase II during the assembly of the pre-replicative complex and with DNA-bound topoisomerase I at the G1/S border. Inhibition of topoisomerase I activity abolishes origin firing. Thus, the two topoisomerases are closely associated with the replicative complexes, and DNA topology plays an essential functional role in origin activation.

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Introduction

The regulation of DNA replication of eukaryotic organisms is mediated by the cell-cycle-dependent assembly and reorganization of specific multiprotein complexes on the origins of DNA replication. DNA topoisomerases are required for the processes of activation of the ori C of *Escherichia coli* (Kornberg, 1984) and of the origins of SV40 (Halmer *et al.*, 1998), BPV (Hu *et al.*, 2006) and EBV genomes (Kawanishi, 1993); furthermore, *in vitro* DNA synthesis with a

Saccharomyces cerevisiae system requires negatively supercoiled DNA and the action of DNA topoisomerase I (topo I) (Mitkova *et al.*, 2005), and negatively supercoiled DNA is also required for the binding of the *Drosophila* ORC (Remus *et al.*, 2004). Thus, the modifications of the specific protein–DNA interactions occurring at human replication origins may entail DNA topoisomerase-induced modulations of the topological state of the origin DNA. We addressed this question for the origin of DNA replication located at the 3′ end of the human gene for lamin B2, for which we have described the precise cell-cycle-modulated interactions with some components of the replicative complexes (Abdurashidova *et al.*, 2003).

The sites of action of topoisomerases may be detected *in vivo* by freezing specifically and reversibly the intermediates of the reaction they catalyze using specific poisons: camptothecin (CPT) for topo I and the etoposide VP16 for topo II; these drugs forbid the reformation of the cleaved phosphodiester bonds and leave the enzymes covalently bound to the 3′-phosphate (topo I) or 5′-phosphate (topo II) of the cleaved bond, freezing the so-called ‘cleavage complex’ (Burden and Osheroff, 1998; Pommier *et al.*, 1998). By coupling the treatment with the appropriate poison with ligation-mediated PCR (LM-PCR) analysis (topo I; Strumberg *et al.*, 2000; Mueller *et al.*, 2001) or terminal transferase-dependent PCR (TD-PCR) analysis (topo II; Komura and Riggs, 1998), we localized the precise positions of the two topoisomerases *in vivo*, in a 1500-bp region comprising the lamin B2 origin, throughout the cell cycle. We also demonstrated that topo I and II are members of the human origin binding complex and established that topo I activity is essential for replicon firing. Furthermore, in a parallel *in vitro* approach, we observed different modes of interaction of topo I and II with the origin.

Results and discussion

Presence of active topo I and II at the origin

To gain insights into the role of DNA topology for origin function, we investigated the behavior of topo I, which is required for origin function in viruses and yeast. To identify the possible presence of active topo I in the origin area, asynchronous HeLa cells were treated for 1 min with increasing concentrations of CPT. DNA was extracted and analyzed by LM-PCR with appropriate primers for the upper and lower strands (see Materials and methods). This analysis (Figure 1A) identifies the positions of the 5′OH residues arising from topo I action on either strand in the area covered *in vivo* by the replicative complexes. Only two sites are cleaved by the enzyme, one on the upper strand between nucleotides 3890 and 3891, and the other on the lower strand between nucleotides 3956 and 3957 (see Figure 1C).

To rule out the possibility that these cuts derive from secondary effects of the CPT treatment, such as a possible disruption of the chromatin and replicative complex structure, we performed a photo-footprinting analysis of the origin

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Figure 1 Interaction of topo I and II with the lamin B2 origin *in vivo*. (A) LM-PCR-mediated analysis of the topo I-DNA cleavage complexes induced by 1 min treatment of asynchronously growing HeLa cells with increasing concentrations of CPT (1, 10 and 100 nM, lanes 3–5 and 1 μ M, lanes 6 and 12) or with 10 μ M gimatecan (lane 7). Lanes 2 and 11: control genomic DNA from untreated cells; lanes 1 and 10: Maxam–Gilbert sequencing reaction. TD-PCR analysis of the distribution of UV photoproducts along the origin region in UV-irradiated HeLa cells treated for 1 min with 1 μ M CPT (lanes 9 and 14) or left untreated (lanes 8 and 13). (B) TD-PCR-mediated analysis of the topo II-DNA cleavage complexes induced by treatment of asynchronously growing HeLa cells with 10 nM VP16 (lanes 3 and 10); lanes 2 and 9: control genomic DNA from untreated cells; DNA immunoprecipitated with anti-topo II antibody from cells subjected (lane 4) or not subjected (lane 5) to VP16 treatment; TD-PCR analysis of the distribution of UV photoproducts along the origin region in UV-irradiated HeLa cells treated for 1 min with 10 nM VP16 (lanes 7 and 12) or left untreated (lanes 6 and 11). (C) Summary of topo I and II *in vivo* cleavage sites at the lamin B2 origin area involved in the replicative complexes. Leading strand start sites are indicated by arrows and DNA cleavage complexes by filled triangles.

area in cells treated or not treated with CPT. Irradiation of the cells with short UV pulses using a femtosecond laser source induces DNA damage (photoproducts or protein–base cross-links). As shown in lanes 8–9 and 13–14 of Figure 1A, the pattern of photo-footprinting on both strands was not perturbed by CPT, with the single conspicuous difference of a band related to the frozen topo I cleavage complex.

The selection of the cleaved phosphodiester bond is an intrinsic property of the enzyme, as (as shown in lane 7 of Figure 1A) the same cleavage *in vivo* is detected upon treatment with gimatecan, a CPT derivative modified at the 7-carbon and displaying a different electronic structure.

We also investigated the involvement of topo II. In the past, a topo II cleavage site was mapped in a 2-kb region containing the lamin B2 origin (Lagarkova *et al*, 1998). We treated asynchronous HeLa cells with VP16 for 1 min and mapped the position of the topo II-mediated cleavage complexes by TDPCR. The results in Figure 1B show the interaction of topo II with two sites, both in the area covered by the pre-replicative complex. One site occurs on the upper strand between nucleotides 3914 and 3915 and the other site is located on the lower strand between nucleotides 3940 and 3941 (see Figure 1C). To prove that the observed cuts map the sites of topo II interaction, DNA extracted from VP16-treated cells was digested with λ -exonuclease and immunoprecipitated with anti-topo II antibody. TD-PCR analysis of the precipitated DNA (see Figure 1B, lanes 4 and 5) using primer set D (that explores the cut in the lower strand) identifies the same stops, in agreement with the property of topo II to be covalently bound to the 5' end of DNA, thus making it resistant to λ -exonuclease action. Also in the case of topo II

(see Figure 1B, lanes 6, 7 and 11, 12), photo-footprinting analysis did not indicate any gross disruption of the origin structure caused by the topo II block, with the exception of the presence of the topo II-mediated cleavage. For both topo I and II, no cleavages were observed in the absence of poison treatment (see Figure 1A, lanes 2 and 11, and Figure 1B, lanes 2 and 9).

Thus, both topoisomerases interact with the origin area, within the sequence covered by the replicative complexes (Dimitrova *et al*, 1996; Abdurashidova *et al*, 1998; Paixao *et al*, 2004), close to and ahead of the start sites, on the templates for leading strand synthesis.

Sequence dependence of the selection for the cleaved bonds

We investigated whether the selection of cleaved origin residues by these enzymes is dictated by direct enzyme/DNA sequence recognition or by other factors. When pure topo I was incubated with origin DNA, CPT induced in the lower strand a single cut in exactly the same position as the one observed *in vivo* (see Figure 2A, lane 3 and WT row of Figure 2C). The same result was obtained with different CPT derivatives (see Figure 2A, lanes 5 and 6). In the case of the upper strand, four topo I-mediated CPT-induced cleavages were observed, of which one coincides with the *in vivo* cleavage mapped on the same strand (see Figure 2A, lane 10). All the cleavages observed *in vitro* are topo I-mediated, as incubation of origin DNA with CPT in the absence of topo I showed no cleavages (see Figure 2A, lanes 1 and 8).

The replacement of a 10 T stretch, shown to spontaneously acquire *in vitro* unusual, probably triple-stranded, structures

Figure 2 Interaction of topo I and II with the lamin B2 origin *in vitro*. (A) Detection of the *in vitro* topo I cleavages stabilized on the lower strand by CPT (lane 3), 7-[CH₂-Tris] CPT (lane 5) or gimatecan (lane 6), and on the upper strand by CPT (lane 10); lanes 7 and 12: Maxam-Gilbert sequencing reactions; the position of the cleavages also present *in vivo* is indicated by an asterisk. (B) Effect of base substitution mutations in the lamin B2 origin on topo I-mediated cleavage. (C) Sequence of the origin portion covered by the replicative complexes; the position of substituted bases is highlighted; the position of *in vitro* topo I-cleavable complexes is indicated by filled triangles; the asterisks indicate the position of the topo I cleavages also present *in vivo*. (D) Detection of the *in vitro* VP16-induced topo II cleavages introduced by the enzyme alone (lanes 1–5) or by topo II as part of a complex with nuclear proteins (lanes 6–14); lane 9: the origin DNA incubated with the nuclear extract and VP16 was immunopurified using anti-topo II antibody; black vertical bars indicate the region protected *in vivo* by the origin binding proteins (OBP) as determined by λ-exonuclease digestion; lanes 10 and 14: Maxam-Gilbert sequencing reactions.

(Kusic *et al*, 2005) and located close to the *in vivo* topo I site on the upper strand, with a grossly modified sequence, abolished the two nearby *in vitro* cleavage sites but not the far away ones (see Figure 2B, lanes 4 and 13, and C). The interaction with the lower strand was abrogated instead by replacement of six nucleotides comprising the topo I binding site (see Figure 2B, lane 7, and C). At the same time, this mutation did not affect any of the upper strand cleavage sites (see Figure 2B, lane 10). Therefore, in spite of the lack of sequence similarity between the mapped topo I cleavages, it is obvious that the enzyme has a clear affinity for a given region.

Topo II, instead, is not addressed to its specific sites by direct enzyme/sequence recognition: the pure enzyme,

incubated with origin DNA in the presence of VP16, introduced cuts without obvious sequence preference (see Figure 2D, lanes 1–5).

Considering the precise *in vivo* localization of topo II on origin DNA in asynchronous cells, we investigated if the topo II–lamin B2 interaction might be influenced by other nuclear proteins. We took advantage of the fact that we could build *in vitro* a specific multiprotein complex on origin DNA, which has a definite electrophoretic mobility shift (data not shown) and covers the area from nt 3851 to 4007 (as demonstrated by a λ-exonuclease protection assay, see arrows in Figure 2D), similar to the area protected *in vivo* in the middle of G1 (Abdurashidova *et al*, 1998). In contrast to the results

obtained with pure topo II, the origin DNA incubated in the presence of the nuclear extract exhibited completely different polymerase stops. In the presence of SDS, but regardless of VP16 addition (in good agreement with previous *in vitro* data showing that denaturation of topo II stabilizes the cleavage complex; Lee and Hsieh, 1992), origin DNA displays, on both upper and lower strands, the same cleavage pattern as the *in vivo*-mapped topo II cutting sites (see Figure 2D, lanes 6, 7, 11 and 12). These cuts are indeed due to topo II, as the same cleavages were detected in protein–DNA complexes immunoprecipitated with an anti-topo II antibody (Figure 2D, lane 9). In good agreement with the lack of sequence specificity of pure topo II, and the fact that it can only recognize the same *in vivo* sites as part of a complex with other proteins, replacement of 10 nucleotides near the lower strand topo II cleavage site (nt 3943–3952) did not abolish any of the topo II cuts introduced by the nuclear extract on either strand (data not shown). Thus, in contrast to topo I, topo II has no

intrinsic sequence affinity for the lamin B2 origin, but is directed at precise sites by other proteins present in the nucleus.

Interaction of topoisomerases with the origin along the cell cycle

To investigate the cell-cycle-dependent interaction of topo I with the origin, cells were collected in M, early G1, middle G1, late G1 (mimosine block), G1/S border–early S (aphidicolin block) and S phase, and subjected to CPT treatment and LM-PCR analysis using the primer sets shown in Figure 3A. The results in Figure 3B show that topo I is clearly present on the origin, at the already identified sites, in M and early G1, leaves it before the middle of G1 and reappears again at the same sites in late G1–G1/S border, finally to leave the origin again in S, when the lamin B2 origin has fired. The presence of the CPT-induced cuts in mimosine-arrested cells (late G1) shows that the enzyme is acting before synthesis starts, as

Figure 3 Topo I interacts with the lamin B2 origin in a cell-cycle-dependent manner, and is a member of the origin binding complex. (A) Localization and orientation of the primer sets in the analyzed region; the positions of the detected topo I–DNA complexes are indicated by vertical arrows. (B) LM-PCR-mediated detection of CPT-induced topo I cleavage complexes on the lower and upper strands in different moments of the cell cycle; G, *in vitro* DMS-treated genomic DNA. (C) Identification of the presence of topo I on the CPT-induced cleavage sites and interaction of the enzyme with Orc2p; in the upper portion are shown the positions of the primers utilized (arrows) relative to topo I cutting sites; HeLa cells subjected to CPT treatment were crosslinked or not with DSP, lysed and the DNA was immunopurified with anti-topo I, anti-Orc2p or unrelated antibodies; the lower portion shows the PCR analysis of untreated genomic DNA (lanes 1 and 17), of the DNA immunopurified with anti-topo I antibodies (lanes 2–4), with anti-Orc2p antibodies (lanes 13–16) or with unrelated antibodies (lanes 5–12). (D) Topo I co-immunoprecipitates with Orc2p in HeLa nuclear extract: Western blot of proteins immunoprecipitated with anti-Orc2p antibody and assayed with anti-topo I or anti-Orc2p antibodies. (E) Formaldehyde crosslinking shows that both topo I and Orc2p associate with the lamin B2 origin in late G1; the DNA from formaldehyde crosslinked HeLa cells was immunopurified using anti-topo I or anti-Orc2 antibodies or pre-immune serum and subjected to competitive PCR analysis; B48, origin region; B13 non-origin region.

this drug does not allow entry in S, whereas aphidicolin may not cause an absolute block of initiation.

The cell-cycle dependence of the origin interaction with topo I is also observable at the same sites and at the same cell-cycle moments in human fibroblasts, synchronized in G0 by serum starvation, and in HeLa cells collected in M by nocodazole treatment, released into G1 in the absence of any drug (see Supplementary Figure 2).

We extended our analysis of the presence of topo I far from the replicative complex area to a total length of 1500 bp (see Figure 3A for the position and direction of all primer sets used). On the left side, no other cleaved bond was observed on either strand, whereas on the right side only one further site was found to be present in S phase on the lower strand, far from the area covered by the replicative complexes, between nt 4335 and 4340 (primer set B). This corresponds to the region located on the 5' side of the template of the housekeeping TIMM13 gene, just downstream of the promoter. It is conceivable that the action of topo I at this site is related to transcription.

Thus, the *in vivo* interaction of this enzyme with the origin within the replicative complex area appears to be focused by its intrinsic affinity towards this region in the close neighborhood of the start sites of synthesis.

To ascertain whether the CPT-induced cleavages are indeed topo I-mediated, we designed the experiment reported in Figure 3C; the DNA of the cells synchronized in late G1 with mimosine and treated or not treated with CPT was isolated and digested with a restriction enzyme, yielding a fragment encompassing the two sites cut *in vivo* inside the area covered by the replicative complex. The DNA-protein complexes derived from the CPT-treated or not treated cells were subjected to immunoprecipitation with anti-topo I antibodies; the DNA preparations were subjected to PCR with the primer sets located at the positions shown in Figure 3C. If the cuts were actually caused by topo I, we should find in the precipitate the fragments close to the sites cut *in vivo*. As expected, in control untreated cells, total DNA gave the three expected PCR products (one encompassing and two not encompassing the cleaved bond) (see Figure 3C, lane 1). In contrast, in the presence of CPT, the anti-topo I antibody selectively immunoprecipitated only the two fragments on either side of the cleaved bonds, in good agreement with the property of topo I to be covalently bound to the 3' end of the DNA (see Figure 3C, lane 2).

Considering the presence of topo I in late G1–G1/S border within the area covered by the replicative complex, it was tempting to assume that topo I acts as part of the origin binding complex. To investigate if this is indeed the case, we treated the cells synchronized in late G1 with both CPT and the protein-protein crosslinking agent dithiobis-(succinidyl-propionate) (DSP), together or separately. The DNA-protein complexes were then isolated, immunoprecipitated with anti-Orc2p antibody and analyzed by PCR with the same primers shown in Figure 3C. The results reported in lanes 13–16 of Figure 3C highlight that anti-Orc2p antibody does not precipitate any DNA when CPT and DSP treatments were performed separately (see lanes 13 and 15). In contrast, in the case of the combined treatment, antibody against Orc2p selectively immunoprecipitated exactly the same DNA fragments shown to be covalently bound to topo I (see Figure 3C, lane 14), demonstrating that topo I and Orc2p are interacting

either directly or through some intermediate protein, being in any case both members of the complex. The interaction of the two molecules is confirmed by the observation that when a nuclear extract was precipitated with the anti-Orc2p antibody, the precipitated proteins were shown to contain topo I (see Figure 3D). Finally, a chromatin immunoprecipitation assay performed on late G1-arrested cells with either anti-Orc2p or anti-topo I antibody yielded a clear enrichment for the lamin B2 origin sequence (Figure 3E), confirming that both proteins are found on the origin at this moment of the cell cycle.

Interestingly, as shown in Figure 3C, both topo I residues located on the lower and upper strands interact with Orc2p, indicating that this ORC member interacts with topo I on the upper and lower strands, although we cannot tell whether the same or two different Orc2p molecule(s) interact(s) with the topo I molecules located on either side of the start site of synthesis.

The experiments reported in Figure 3C–E allow us to conclude that topo I is closely associated with the human origin binding complex on lamin B2.

In summary, topo I binds selectively the templates of leading strand synthesis, each at one precise bond ahead of and close to the start site of DNA synthesis. The binding occurs in M, early G1 and late G1–G1/S border. At this last moment, topoisomerase I is adjacent to Orc2p. It is tempting to consider the interaction close to the start site (in association with Orc2p) correlated with the topological demands of the origin for its proper and timely function, considering that the origin fires at the onset of S.

Analysis of the cell-cycle-dependent behavior of topo II, as reported in Figure 4A–C, shows that the enzyme is bound at the indicated sites in M and in the middle of G1. When we extended our analysis to a total of 1500 bp with different primer sets (see Figure 4B), we identified two stops with the primer set G (Figure 4C). Besides the cleavage located at the origin, we also observed another cleavage, constant throughout the cell cycle and located over 200 bp away from the origin-bound topo II molecule. When with the same primer set G we analyzed DNA immunoprecipitated with anti-Orc2p antibody from the cells collected in the middle of G1 treated with VP16 and DSP together or separately, we detected just one stop corresponding to the origin-bound topo II only when VP16 and DSP were used together, showing that just this topo II molecule interacts with Orc2 (Figure 4D). Therefore, only this topo II molecule is a member of the human origin binding complex, in agreement with the observation that only this site is comprised within the area covered *in vivo* by the replicative complexes.

Thus, within the 1500-bp explored area, topo II is bound *in vivo* only to three sites: one site is 170 bp removed to the left of the replicative complex area and could correspond to a scaffold attachment region. The other two sites lie very close to the start sites of bidirectional synthesis. The enzyme is bound to these two sites in mitotic chromosomes, like topo I; preliminary data indicate that these two enzymes are not bound contemporaneously to origin DNA: simultaneous treatment of asynchronous cells with CPT and increasing concentrations of VP16 followed by analysis of the induced cuts using primer set D showed the presence of both the topo I and II stops in the lower strand, an outcome possible only if the enzymes are present in different molecules, as we use poison concentrations giving maximal effect (data not

Figure 4 Topo II interacts with the lamin B2 origin in a cell-cycle-dependent manner, and is a member of the origin binding complex. (A) TD-PCR-mediated detection of VP16-induced topo II cleavages on the upper strand; G, *in vitro* DMS-treated genomic DNA. (B) Localization and orientation of the primer sets in the region analyzed; the positions of the detected topo II-DNA complexes are indicated by vertical arrows. (C) TD-PCR-mediated detection of VP16-induced topo II cleavages on the lower strand; G, *in vitro* DMS-treated genomic DNA. (D) TD-PCR analysis of DNA immunoprecipitated with anti-Orc2p (lanes 3–6) or unrelated antibodies (lanes 1 and 2) from HeLa cells synchronized in the middle of G1, treated with VP16, DSP or both; topo II binding sites at or outside of the origin are indicated with one or two asterisks respectively; G, *in vitro* DMS-treated genomic DNA.

shown). In view of the demonstrated presence of topo I on the origin in early G1, we surmise that topo II binds the origin in early mitosis, possibly contributing to the packing of metaphase chromosomes, and topo I binds towards the end of M. Topo II is then present at the origin in the middle of G1, where it interacts with Orc2 inside the pre-replicative complex, pointing towards a role of topo II in the assembly and reorganization of the G1 pre-replicative complex.

In all the experiments described here, we have invariably observed the presence of topo II on one strand only, and could never obtain evidence for the interaction with the complementary strand, 4 bp removed, which is a described property of this homodimeric enzyme. This observation has many precedents, as conditions in which the enzyme remains

bound, after poison blockage, to one strand only have been described in several instances (Muller *et al*, 1988; Lee *et al*, 1989). Furthermore, one has to consider that the action of topo II on the origin that we investigated invariably occurs in the context of a multiprotein complex, *in vivo* or *in vitro*, and that protein–protein interactions may be at the base of the preference for one strand. Also, it is tempting to hypothesize that the origin forms a loop whereby the two subunits of the same topo II molecule interact with the two sites of the templates for the oppositely moving leading strands.

Functional consequences of the topo I block

The presence of topo I just ahead of the start sites of synthesis on the templates of the leading strands immediately before

initiation of synthesis points to a direct role of this enzyme in origin firing; does synthesis start if topo I action is inhibited by CPT? It has already been demonstrated that CPT treatment inhibits overall DNA initiation in human cells (Kaufmann *et al*, 1991; Wang *et al*, 2004). Yet, those experiments could not distinguish between an actual inhibition of initiation and a situation in which replication is initiated but the growing forks are stopped when they hit the cut caused by the drug at a short distance from the start site (and we know that topo I is indeed located on the template close to the start sites). In order to analyze in detail whether the drug forbids specifically the initiation at the lamin B2 origin, and not just the elongation at some distance from the start sites, we performed the experiments reported in Figures 5 and 6. If replication starts (see the scheme in Figure 5) and is then necessarily blocked by the topo I cleavage complex, the sequence immediately adjacent to the start site should exhibit a double-stranded blunt end; such end, following DNA extraction, isolation and phosphorylation, could be specifically detected by modified LM-PCR analysis (Zaret, 2005), in which the initial primer extension step is eliminated and the double-stranded DNA directly ligated to the asymmetric linker; actually, the presence of such ends produced by

replication run-off was previously demonstrated by the study of the mechanism of CPT inhibition of DNA synthesis in asynchronous cultures (Strumberg *et al*, 2000). As shown in Figure 5, this is not our case, and we have to conclude that synthesis has not initiated. In order to check that our conditions are adequate to detect the blunt-ended cuts, we treated DNA with micrococcal nuclease that is known to produce double-stranded breaks in the nucleosome linker DNA: LM-PCR treatment of the fragments allowed the identification of such breaks (see Figure 5, last gel of panel B). We also considered the possibility that the synthesis might have started but aborted before reaching the topo I site. To test this possibility, we incubated the isolated DNA with DNA polymerase to allow the completion of synthesis from any possible recessed 3'OH end on the origin DNA, but the results were still negative. No LM-PCR was obtained when the experiment was performed in the presence of caffeine, which allows the formation of the cleavage complex but abrogates checkpoint response (Sarkaria *et al*, 1999), indicating that the failure to produce double-stranded breaks is not due to inhibition of DNA synthesis by checkpoint pathways. Topo I action is essential for synthesis start.

Figure 5 Inhibition of replicon activation following stabilization of topo I; 1. Stabilized topo I cleavage complexes at the lamin B2 origin do not lead to replication run-off. (A) Schematic representation of topo I-mediated single-strand breaks (SSB) induced by CPT (left side) or of the replication-dependent formation of double-strand breaks (DSB) (right side). (B) modified LM-PCR detection of the presence of SSB and DSB in the ori region; cells were collected at late G1 with mimosine, 1 μ M CPT was added and they were released from mimosine for the indicated time; the same experiments were performed in the presence of 5 mM caffeine as indicated; DNA was isolated (see Materials and methods) and subjected to modified LM-PCR with (first two gels) or without (last two gels) first primer extension, as described (Strumberg *et al*, 2000). If no primers were added, no blunt-ended duplexes were formed, the linker ligation could not operate and no amplification was obtained, even in the presence of DNA polymerase added to complete possible recessed duplexes. If, after 30 min exposure to CPT, this drug is removed and the cells incubated for further 10 min, the topo I-induced cut is completely abolished (last lanes of first two gels). The last gel shows the detection of double-stranded blunt ends produced by micrococcal nuclease treatment of cells or of naked DNA utilizing the modified LM-PCR procedure (Zaret, 2005) with primer set A.

Figure 6 Inhibition of replicon activation following stabilization of topo I; 2. Short nascent DNA of 0.6–1 kb was isolated by denaturing gel electrophoresis from HeLa cells collected at late G1 (mimosine synchronization) and pulse-labeled with BrdU in the presence of CPT (see Materials and methods). The top portion schematizes the expected distribution of BrdU-containing DNA (crosses and dots) if the origin fires in the absence (leftmost bubble) or presence of CPT (central bubble) or does not fire (rightmost bubble). The lower portion shows the quantification by competitive PCR analysis of the abundance of origin (B48) or non-origin (B13) sequences in the total nascent DNA and in DNA immunopurified with anti-BrdU antibody. Cells released from the mimosine block for 5 min enter S phase as shown by the comparable enrichment in origin (B48) over non-origin (B13) sequences of total and BrdU-labelled nascent DNA (left panel). Conversely, in the presence of CPT, the origin does not initiate, as shown by the absence of origin enrichment in the BrdU-containing DNA (the antibody efficiently precipitates nascent DNA molecules containing a single BrdU residue: Supplementary Figure 4).

This conclusion was also checked in another way (Figure 6). Cells were collected in late G1–G1/S border, treated with CPT and released in S phase in the presence of CPT and BrdU. If, in the presence of CPT-stabilized inactive topo I, DNA synthesis may start and stop at the topo I cleavage complex, this short nascent DNA will be labelled with BrdU. Instead, if the origin does not fire, upon removal of both CPT and BrdU, reversal of the CPT-stabilized covalent complex should allow the origin to initiate DNA synthesis, since, as shown in the last lanes of the gels of Figure 5, release from the CPT block is complete by 10 min. As shown in Figure 6, an analysis of the presence of origin DNA in the nascent fraction (by measuring the relative abundance of the origin-containing B48 fragment and the non-origin B13 one) shows that the origin sequence is strongly reduced in the BrdU-containing DNA when the cells are treated with CPT; on

the contrary, as shown in the first panel of Figure 6, if no CPT was added, the origin containing fragment was significantly enriched in the nascent BrdU-containing DNA; conversely, in cells maintained in the presence of mimosine, the origin could not initiate (see Supplementary Figure 3). The inactivation of topo I has forbidden synthesis start.

Hence, topo I action is required for replication initiation at the lamin B2 origin, and the fine control of DNA topology seems to be essential for origin function.

Role of DNA topology in origin specification and function

The existence of replication origins in metazoans, from which bidirectional forks reproducibly issue at each cell division, raises the question of the factors that specify a chromosomal region for this function. No obvious sequence similarity has

Figure 7 Summary of the mapped protein–DNA interactions at the lamin B2 origin. The cartoon summarizes the interaction of active topo I and topo II molecules with the origin sequence along the cell cycle demonstrated in the present work and reports also the data previously obtained for the interactions of Orc1p, Orc2p and Cdc6p with the same sequence (Abdurashidova *et al*, 2003). The interaction of the two enzymes with the ORC complex is also shown.

been observed among metazoan origins, even within the same organisms, and the ORC complex does not show any sequence specificity; yet all different origins assemble an ORC-containing replicative complex, the same origin works

in different cells of the same organism and can still assemble the replicative complex and fire when transferred to other chromosomal locations, and even when transferred to the chromosomes of a different organism (DePamphilis, 2006 and

references therein). Which role does the topological status of the origin area play?

We have addressed this question with the present work and, in Figure 7, we summarize our findings on the interactions of DNA topology-modifying enzymes with the lamin B2 origin. In the figure, we also report the knowledge, obtained in the course of our previous investigations, on the position within the origin area of some components of the replicative complexes in different moments of the cell cycle (Abdurashidova *et al*, 1998, 2003).

Both topoisomerases interact with the sequence involved in the replication complex transactions binding the templates of leading strand synthesis, closely ahead of the start sites (topo II being closer). In M, the lamin B2 origin appears free of extensive protein–DNA interactions (Abdurashidova *et al*, 1998). Yet, both topoisomerases are present in the origin area, although apparently in different moments of M. In early M, topo II could concur in creating the close packing of metaphase chromosomes. At later stages of M, topo II leaves the origin area and is replaced by topo I that remains bound in the early portion of G1 possibly to help in unpacking chromosomes into their interphase structure.

The entry into G1 is the moment when we begin to observe the formation of a protein–DNA complex on the origin (data not shown). On another metazoan origin, it was actually shown that members of the replicative complex begin to assemble in early G1, (Dimitrova *et al*, 2002), but, in our case, we do not yet know which proteins belong to this early G1 complex, besides topo I. It appears plausible that this initial build-up of the pre-replicative complex could be aided by the presence of topo I. What determines the selection of this specific fraction of the genome to assemble a functional specific origin? Although the basis for origin identification in metazoans remains mysterious (Aladjem *et al*, 2006), the intrinsic sequence affinity that we have observed for the interaction of topo I with the origin could provide an important contribution to origin selection.

As G1 progresses, a further exchange between topo I and II accompanies the complete assembly of the pre-replicative complex. In Figure 7, we report, besides the position of topo II on both strands, also the precise position of two ORC proteins and Cdc6 on the lower helix only, these being the only proteins and strands explored (but certainly all the ORC and other proteins are bound, probably interacting with both strands). The area bound to topo I earlier in G1 now gets occupied by Orc2p, further supporting the idea that topology-modifying events may be required for the formation of the pre-replicative complex. We propose that topo II is brought to the origin by interaction with other specific proteins, and then cooperates in assuring the structural modifications that lead to the formation of the pre-replicative complex. The direct interaction of this enzyme with Orc2p when bound to the origin DNA is a witness of the involvement of a topology-modifying agent in this process.

In late G1, as the pre-replicative complex is fully assembled, a third exchange between topo II and I takes place. Topo I reappears ahead of the start sites replacing and still closely interacting with the Orc2p; when the origin is poised for synthesis start, Orc1p and Cdc6p leave the pre-replicative complex. A restructuring of the replicative complex occurs after origin firing that includes the departure of topo I. In S, we witness the displacement of Orc2p closer to

the now inactive start site, at a position that, in middle G1 and M, was occupied by topo II. The subsequent reformation of the mitotic chromosomes is accompanied by the departure of the ORC proteins and the renewed recruitment of topo II and topo I.

Altogether, the two topoisomerases are never acting in the replicative complex area at the same time, so that they seem to specialize their functions in the context of topology modulation along the origin activation–deactivation process, topo II seemingly involved in pre-replicative complex assembly and topo I in origin firing. The high resolution of the mapping of topoisomerases and Orc2p at the lamin B2 origin highlights the dynamic interplay between the ORC and topology-modifying enzymes throughout the cell cycle.

The direct involvement of topo I via interaction with the relative origin-specific binding protein has been well established for the activation of the origins of the viral genomes of SV40 (Halmer *et al*, 1998), EBV (Kawanishi, 1993) and BPV (Hu *et al*, 2006). We demonstrate here that the same enzyme is essential for firing of the lamin B2 origin in close interaction with ORC.

In conclusion, our results demonstrate that topo I and II are closely interacting with the replicative complexes, at least at the lamin B2 origin, and are thus likely to play an essential role in the regulation of DNA replication in human cells, confirming the crucial importance of DNA topology for the origin activation–deactivation cycle. Their action is partially determined by DNA sequence, hinting at a possible significant contribution in origin specification, but also directed by other nuclear proteins, leading to complex, dynamic, protein–protein and protein–DNA interactions, of which the intriguing exchanges and contacts with Orc2p are just one element.

Materials and methods

Drugs and chemicals

All drugs and chemicals were purchased from Sigma, unless otherwise stated. The CPT derivatives CPT-7[CH₂-Tris] and gimatecan were synthesized according to the published protocols (Dallavalle *et al*, 2001; Wadkins *et al*, 2004).

Cell culture and synchronization

HeLa cells were cultured in D-MEM/F-12 (1:1) with GlutaMAXTM I supplemented with 10% fetal calf serum (Invitrogen) and 50 µg/ml gentamicin. Exponentially growing cells were arrested in M phase by incubation with nocodazole at 50 ng/ml final concentration. Mitotic cells were recovered by mechanical shake-off 16 h later, washed off nocodazole and replated in nocodazole-free medium. At different points of the G1 phase, unattached cells were washed away and only cells firmly attached were used. To arrest cells in the late G1 or G1/S border–early S, M cells were released in complete medium for 5 h and then incubated with either mimosine at 5 mM final concentration for 24 h (late G1) or with aphidicolin at 5 µg/ml for 16 h (G1/S border–early S). Cells synchronized at G1/S border–early S were then washed three times with complete medium and released for different periods of time to give different S-phase populations. Cell-cycle progression was monitored by FACS (see Supplementary Figure 1).

Mapping of topo I and topo II in vivo

For each experiment, ~10⁷ HeLa cells were incubated with either 1 µM CPT or 10 nM VP16 in complete medium for 1 min, washed twice with PBS containing the same amount of drug and lysed in 250 mM Tris–HCl, pH 8, 25 mM EDTA, 5 mM NaCl, 0.5% SDS and 800 µg/ml proteinase K. To check reversal of drug-induced cleavage, treated cells were washed off the drug and left in complete medium for 10 min. Cell lysates were incubated overnight at 37°C, the DNA

was isolated by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and resuspended in 20 μ l TE buffer (1 mM EDTA and 10 mM Tris-HCl pH 7.5). The topo I cleavage sites were detected by LM-PCR as previously described (Strumberg *et al*, 2000; Mueller *et al*, 2001). The topo II cleavage sites were detected by TD-PCR (Komura and Riggs, 1998). The primer sets used for LM- and TD-PCR have been described elsewhere (Dimitrova *et al*, 1996; Abdurashidova *et al*, 2000).

UV laser photo-footprinting

Asynchronous HeLa cells growing in 5-cm diameter Petri dishes were treated for 1 min with either 1 μ M CPT or 10 nM VP16, washed with PBS containing the same drug concentration and UV laser irradiated at two wavelengths (Russman *et al*, 1998) obtained by frequency conversion of infrared femtosecond pulses produced by a Ti:sapphire system. Second and third harmonic light pulses at 400 and 266 nm, of duration 120 and 200 fs, pulse energy of 25 and 40 μ J, respectively, were produced in two consecutive BBO crystals and expanded to a diameter of about 60 mm to allow simultaneous irradiation of the surface of the dish. For comparison, HeLa cells not treated with the drugs were irradiated under the same conditions. The cells were immediately lysed after irradiation and the DNA isolation and TD-PCR were performed as described.

BrdU labelling

HeLa cells synchronized with mimosine were treated with 1 μ M CPT in complete medium for 30 min, washed in complete medium containing 1 μ M CPT and pulsed with 50 μ M BrdU (Abcam) in the presence of CPT for 30 min. The cells were first washed off BrdU, and then CPT and left in complete medium for 5 min. As a control, late-G1 synchronized cells were incubated with BrdU for 30 min in the presence or absence of mimosine, washed and released in complete medium for 5 min. The cells were then collected, resuspended in PBS containing 10% glycerol and lysed for 10 min in the wells of a 1.2% alkaline agarose gel immersed in alkaline running buffer (50 mM NaOH, 1 mM EDTA). The gel was run for 16 h at 2 V/cm and the nascent DNA of size 0.6–1 kb was isolated from the gel using a Qiagen gel extraction kit. BrdU-enriched sequences were immunoprecipitated using anti-BrdU antibody (Abcam), as described by the manufacturer. The isolated nascent DNA was analyzed by competitive PCR. The quantification of the abundance of two different sequences was performed as described previously (Diviacco *et al*, 1992).

Isolation of topo–DNA covalent complexes

Topo–DNA complexes were isolated by using the *in vivo* complex of enzyme (ICE) bioassay (Zhang *et al*, 2004). Cells ($\sim 5 \times 10^7$) were incubated with 1 μ M CPT or 10 nM VP16 for 10 min or left untreated. Immediately after drug treatment, cells were lysed in 1% sarkosyl in TE and lysates were layered on top of a CsCl solution (1.5 g/ml density) and centrifuged at 70 000 r.p.m. for 5 h at 20°C. The cellular DNA was collected, washed twice in TE buffer (10 mM Tris-HCl pH 8 and 1 mM EDTA) and twice in either exonuclease III or λ -exonuclease reaction buffer. Exonuclease III and λ -exonuclease (New England Biolabs) digestion was performed with 200 U exoIII or 5 U λ -exo at 37°C for 1 h.

In vivo crosslinking with DSP

HeLa cells synchronized in the middle of G1 or at late G1 were incubated first with 10 nM VP16 or 1 μ M CPT, respectively, for 10 min, and then immediately subjected to protein–protein crosslinking with DSP (Lomant's reagent, Pierce), as described (Fujita *et al*, 2002). Immediately after crosslinking, the DNA–protein complexes were isolated by ICE bioassay (see above). The cellular DNA was collected, washed twice in TE buffer and subjected to *Bst*NI digestion (100 U, 1 h at 37°C). The protein–DNA complexes were immunoprecipitated using rabbit polyclonal anti-topo I antibody (Abcam), goat polyclonal anti-topo II antibody (Santa Cruz) and mouse monoclonal anti-Orc2p antibody (Stressgen) using the CHIP assay kit (Upstate Biotechnology), as described by the manufacturer. Immunoprecipitated DNA was purified and analyzed by PCR or TD-PCR as described.

Co-immunoprecipitation

HeLa nuclear extract (Cilbiotech) was used for immunoprecipitation with anti-Orc2p antibodies (Stressgen) and the ProFound co-immunoprecipitation kit (Pierce), as described by the manufacturer.

Western blots of the immunoprecipitated material were performed using anti-Orc2p and anti-topo I antibodies and visualized by the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Chromatin immunoprecipitation

Anti-topo I and anti-Orc2p antibodies were used, followed by competitive PCR analysis, on HeLa cells synchronized in late G1 with mimosine by the procedure described before (Paixao *et al*, 2004).

Mapping of topo I and II in vitro

A 216-bp PCR fragment containing the lamin B2 origin was amplified using primers D3 and E3 (Dimitrova *et al*, 1996). PCR fragment (20 ng) were incubated with 1 U of human recombinant topo I (Sigma) in 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 0.1 mM DTT and 100 μ g/ml BSA for 15 min at room temperature, and with 1 μ M CPT for another 5 min. The reaction was stopped by addition of 2% SDS. Topo I was digested overnight with 200 μ g/ml proteinase K, the DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and resuspended in 20 μ l of water. Topo I cleavage was mapped by the primer extension reaction as described before (Gerbi and Bielinsky, 1997). The D3/E3 PCR fragment, 20 ng (see above), were incubated first with 5 U–25 U of human recombinant topo II (USB) in 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 15 μ g/ml BSA and 1 mM ATP for 10 min at room temperature and then for another 5 min more with VP16 at 100 μ M final concentration. The reaction was stopped with SDS 2% final concentration. Topo II was digested overnight with 200 μ g/ml proteinase K, the DNA purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and primer extension was performed as above.

In vitro complex formation

Dignam HeLa 0.4 M nuclear extract 50 μ g (Cilbiotech) was incubated in 22.5 mM HEPES pH 7, 1 mM Tris-HCl pH 7.5, 0.2 mM EDTA, 5 mM MgCl₂, 20 mM KCl, 1 mM ATP, 1 mM DTT and 8% glycerol with 20 μ g of each competitor: poly (dA-dT)-poly(dA-dT) and poly(dG-dC)-poly(dG-dC) (Amersham) at room temperature for 20 min. D3E3 PCR product (20 ng) was added for another 30 min. The reaction was stopped with SDS 2% final. Proteins were digested overnight with 200 μ g/ml proteinase K and the DNA purification and primer extension were performed as above.

λ -Exonuclease protection assay

For mapping the border of the *in vitro* complex, 50 U λ -exonuclease (New England Biolabs) was added to the preformed complex, incubated at 37°C for 3 h and then stopped with 2% SDS final concentration. The DNA was then purified and subjected to primer extension as described above.

Construction of mutated PCR fragments

Primers used to obtain mutated D3/E3 PCR fragments 1–3:

Mut1A: 5' CAGCGCCAGGCCAATGATTTGTAATATACATTTTATGAC TGG 3'
Mut1B: 5' GAACACGCTAGGCATGCATCTTC 3'
Mut2A: 5' CAGCGCCAGGGTACAACACTCCAATAAACATTTTGAT TTTAGG 3'
Mut2B: 5' CCAGTCATAAAATGTATATTACAAATCATTGG 3'
Mut3A: 5' CTCCAATAAACATTTTGATTTTAGTTCTGCCTCTG 3'
Mut3B: 5' GCGCTGACAAAAAGTTTCCAGTCATAAATGTATATT AC 3'

The PCR reaction mixture contained: 5 μ l 10 $\times$ Vent Exo– buffer, 1.5 μ l 100 mM MgSO₄, 1 μ l (10 pmol) of each primer, 1 μ l dNTPs (10 mM each), 5 μ l D3/E3 PCR product (0.1 ng/ μ l), 0.5 μ l Vent Exo– (2 U/ μ l) and H₂O up to 50 μ l.

The amplification conditions were as follows: for primers Mut1A/E3bio: 95°C 5 min, (95°C 30 s, 60°C 30 s, 76°C 1 min) cycled 10 times, (95°C 30 s, 72°C 30 s, 76°C 1 min) cycled 25 times and 76°C 5 min. For primers Mut1B/D3bio: 95°C 5 min, (95°C 30 s, 70°C 30 s, 76°C 1 min) cycled 35 times and 76°C 5 min. For primers Mut2A/E3bio: same as Mut1A/E3bio. For primers Mut2B/D1bio: 95°C 5 min, (95°C 30 s, 60°C 30 s, 76°C 1 min) cycled 35 times and

76°C 5 min. For primers Mut3A/E3bio: same as Mut1A/E3bio. For primers Mut3B/D3bio: same as Mut1B/D3bio.

For each mutation, two PCR fragment were amplified using Vent Exo- as a polymerase in order to obtain blunt ends, each fragment having one of the 5'-ends blocked with a biotin tag. The fragments were purified by PAGE and subsequently on Streptavidin Paramagnetic Beads (Promega) according to the manufacturer's instructions. The fragments were detached from the beads by boiling at 95°C for 5 min in 0.1% SDS, reannealed in a PCR machine, precipitated with ethanol and resuspended in 10 µl H₂O. The two fragments were incubated together overnight at 4°C in a 50 µl ligation mixture containing 23 µl PEG-40, 10 µl of each purified PCR fragment, 5 µl 10 × T4 DNA ligase buffer and 2 µl T4 DNA ligase (400 U/µl) (New England Biolabs). The ligation products

were purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and used for PCR amplification using the D3/E3 primers.

Supplementary data

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

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