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Human topoisomerase I forms double cleavage complexes on natural DNA

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Abstract

DNA topoisomerase I releases torsional stress generated in chromatin during transcription and replication. Usually topoisomerase I is recognized to work as a monomer, but previously we have shown that two molecules can form a dimer-like protein–protein complex on a 'suicide' DNA substrate resulting in a topoisomerase I double cleavage complex. Here we show that during the normal relaxation reaction a considerable fraction of human topoisomerase I formed transient dimers on plasmid DNA too. Recombinant as well as topoisomerase I purified from human cells formed double cleavage complexes within a distance of 12 or 14 nucleotides. When topoisomerase I was isolated from camptothecin-treated HeLa cells, a considerable fraction migrated to the same position as topoisomerase I bearing a covalently bound 12-to-14-mer oligonucleotide. Taken together our data suggest that human topoisomerase I double cleavage complexes are part of the normal catalytic cycle of this enzyme that occur *in vitro* and possibly also *in vivo*.

Keywords: Double cleavage complex; Topoisomerase I; Plasmid DNA; Protein–protein interaction; Camptothecin; Topoisomerase I damage response

DNA topoisomerase I is responsible for many aspects of DNA metabolism. This enzyme can remove positive as well as negative supercoils from DNA that form during transcription and replication. Topoisomerase I seems also to be involved in apoptosis, DNA repair, and DNA recombination [1–5]. The catalytic cycle of the human enzyme (htopoI) is well investigated and, based on the solved crystal structure [6], it is known that htopoI forms a clamp that closes upon binding to a DNA substrate. DNA binding is followed by the nucleophilic attack of an active-site tyrosine that results in the breakage of the DNA backbone and the covalent linkage of the protein to the 3′ end of a deoxynucleotide through a phosphotyrosyl bond. Once the strand is cleaved, a controlled rotation allows the release of the tension, after what the DNA is religated and the enzyme is liberated [7,8]. The transient covalent state of htopoI and DNA during the catalytic cycle is also referred to as the topoisomerase I cleavage complex.

HtopoI has been described to function as a monomer, but newer data indicate that on some occasions it may also form a dimer. Previously, we reported that a covalently trapped cleavage complex could be recognized by an additional htopoI molecule through protein–protein interactions, which led to the formation of a so-called human topoisomerase I double cleavage complex [9]. Such a double cleavage complex consisted of two covalently attached cleavage complexes that were in direct protein–protein contact; the resulting cleavage sites were only 13 nucleotides (nts) apart [9]. HtopoI double cleavage complexes are relatively unstable and may fall apart by the release of one htopoI molecule covalently attached to a 13 nts long fragment from the cleaved substrate [9–11]. *In vitro* studies have shown that the still remaining htopoI cleavage complex is very efficient in religating a foreign DNA strand that can hybridize to the gap generated adjacent to this second cleavage complex. The ligation reaction results in the disso-

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ciation of the remaining cleavage complex. Thereby, the previous double cleavage complex has been repaired. The ligation reaction was called "topoisomerase I induced recombination-like repair" (TIRR) [10].

All these investigations were performed using a so-called "suicide substrate". This DNA substrate contained a htopoI preferential binding sequence and was designed to trap htopoI after the incision step by preventing religation of the liberated 5′-OH end. With such a substrate it could be demonstrated that the trapped enzyme was recognized by another htopoI molecule that incised the DNA about 13 nts apart from the first bound htopoI molecule. Since all these experiments were done with an artificial substrate, it could be argued that double cleavage complexes only form under these particular conditions. Here we show that htopoI readily and very efficiently forms double cleavage complexes after the relaxation of negatively supercoiled plasmid DNA both with recombinant enzyme and with purified htopoI obtained from HeLa S3 cells. Interestingly, when topoisomerase I was isolated from HeLa cells, a considerable fraction migrated to the same gel electrophoretic position as topoisomerase I bearing a covalently bound 13-mer oligonucleotide. This may suggest that double cleavage complexes also form under intracellular conditions.

Materials and methods

Purification of recombinant protein and DNA. Human topoisomerase I was expressed using a recombinant baculovirus (a generous gift from James Champoux) and purified as previously described [9]. Supercoiled pUC19 plasmid was isolated according to [12]. The sequence, synthesis, and purification of L193s and OL26 were as described [9,10].

Detection of htopoI attached to DNA fragments. The indicated amounts of recombinant or isolated endogenous htopoI were incubated with the pUC19 plasmid in a total reaction volume of 20–50 μl in buffer A (12.5 mM Hepes–KOH, pH 7.9, 150 mM KCl, 6 mM $MgCl_2$, 1% glycerol, 0.5 mM EDTA, and 1 mM DTT) at 37 °C unless stated otherwise. Recombinant or HeLa cell-purified htopoI was mixed with plasmid DNA and buffers and incubated for the indicated times followed by a brief incubation (30 s) with 1.25 μM camptothecin (final concentration) or as indicated in the figure legends. The reactions were stopped by the addition of SDS–PAGE loading buffer (final concentration: 1% SDS, 66 mM Tris, 10% glycerol, 143 mM β-mercaptoethanol, and 5 μg/ml bromophenol blue) and heating to 95 °C for 5 min unless stated otherwise. The products were run through a 7.0% SDS–PAGE (until a 100 kDa pre-stained marker band was close the bottom of the gel, 15 mA/gel, ~1.8 mA/cm) (running buffer: 25 mM Tris, 192 mM glycine, and 0.1% SDS) and analyzed by Western blotting using a semi-dry blotter and a PVDF membrane. Subsequently, the membrane was incubated with a murine monoclonal antibody against htopoI (C21, BD-Pharmingen) for 16–20 h at 4 °C in calcium- and magnesium-free (CMF) PBS supplemented with 5% skimmed milk powder (82 mM NaCl, 10 mM Na_2HPO_4, and 10 mM NaH_2PO_4). The membrane was washed and incubated 1 h at room temperature with horseradish peroxidase-coupled anti-mouse antibody (Promega) and detected by enhanced chemical luminescence.

DNA relaxation assay. The relaxation experiments were performed as described above with the exception that CPT was not added and that the reactions were incubated for the indicated time and stopped by the addition of agarose loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 30% glycerol) and heating to 70 °C for 5 min. After cooling down to room temperature the samples were subjected to electrophoresis through a 0.8% agarose gel and visualized by staining with ethidium bromide. Documentation was done using a Kodak Image Station 2000R.

Radioactive labelling of DNA. The cleavage reactions were performed as described above but stopped by incubating 10 min at 70 °C. The protein was digested by the addition of 5 μl of 10 mg/ml trypsin and incubation for 1 h at 37 °C. Subsequently, plasmid DNA was precipitated by the addition of 0.1 volume parts of 4 M NaCl and 4 volume parts of absolute ethanol and incubation for about 20 h at 4 °C. After centrifugation the pellet was resolved in H_2O and incubated with 10 μCi [γ-^{32}P]ATP (Hartmann Analytic) and 20 U T4-polynucleotide-kinase (Fermentas) according to the instructions of the supplier. The reaction mixture was passed over a MoBi Spin S200 column (MoBiTec) to remove free nucleotides. The resulting products were analyzed on a 14% denaturing polyacrylamide gel, dried and detected with a phosphorimager (Storm860, GE Healthcare).

Partial purification of htopoI from HeLa S3 cells. The pellet from HeLa S3 cells (~10 ml) was suspended in 40 ml TE-buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 1 mM leupeptin, and 0.05% aprotinin) and homogenized with a Dounce homogenizer. Then, 40 ml sucrose/glycerol buffer (50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 25% sucrose, 50% glycerol, and 5 mM DTT) was dropwise added under stirring, followed by the slow addition of 10 ml saturated ammonium sulfate (pH 7.0). Subsequently, the extract was spun at 35,000 rpm (218,000*g*) for 3 h at 4 °C in an SW40-Ti rotor (Beckman). The supernatant was adjusted to a total of 1 M ammonium sulfate and spun at 18,000*g* for 10 min. The supernatant was diluted 1:1 in buffer B (25 mM Hepes–KOH, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 17% glycerol, 1 mM EDTA, and 2 mM DTT) supplemented with 1 M NH_4SO_4. The extract was split and loaded onto two phenyl sepharose columns (2.5 ml each) and the run-through as well as the wash fraction (with buffer B) was collected. A withdraw of a small sample the solution was dialyzed against buffer C (10 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 10% glycerol, and 1 mM DTT) for approximately 12 h. The dialyzed solution was passed through a 5 ml HiTrap heparin column equilibrated in buffer C. The column was washed with buffer C supplemented with 500 mM NaCl followed by 1 M NaCl. The fraction eluted at 1 M NaCl was collected, dialyzed against buffer B, and stored at −70 °C. The protein concentration was determined according to Bradford [13].

Camptothecin treatment of HeLa S3 cells. HeLa S3 cells were grown in spinner flasks at 37 °C in DMEM medium supplemented with 10% fetal calf serum. Half of the cells were harvested and the remaining were treated with 10 μM CPT for the indicated times. After harvest the indicated amounts of cells were lysed at 95 °C for 10 min in SDS–PAGE loading buffer and analyzed on a 7.5% SDS–PAGE. Subsequently, a Western blot was performed as described above.

Results and discussion

We have previously shown that htopoI double cleavage complexes were formed on the artificial deoxyoligonucleotide substrate L193s. To investigate whether this phenomenon also takes place on natural DNAs, we set up an assay as depicted in Fig. 1. Recombinant htopoI was pre-incubated with the pUC19 plasmid. During this pre-incubation time htopoI molecules started to relax the supercoiled plasmid; double cleavage complexes should also form. At the moment of CPT addition all htopoI molecules that were just in the process of relaxing DNA, were trapped and covalently bound to DNA. Subsequent addition of SDS denatured and "froze" any cleavage complexes that might have been formed on the DNA. To dissociate the assumed double cleavage complex intermediates from the plasmid, i.e., short DNA strands with attached htopoI

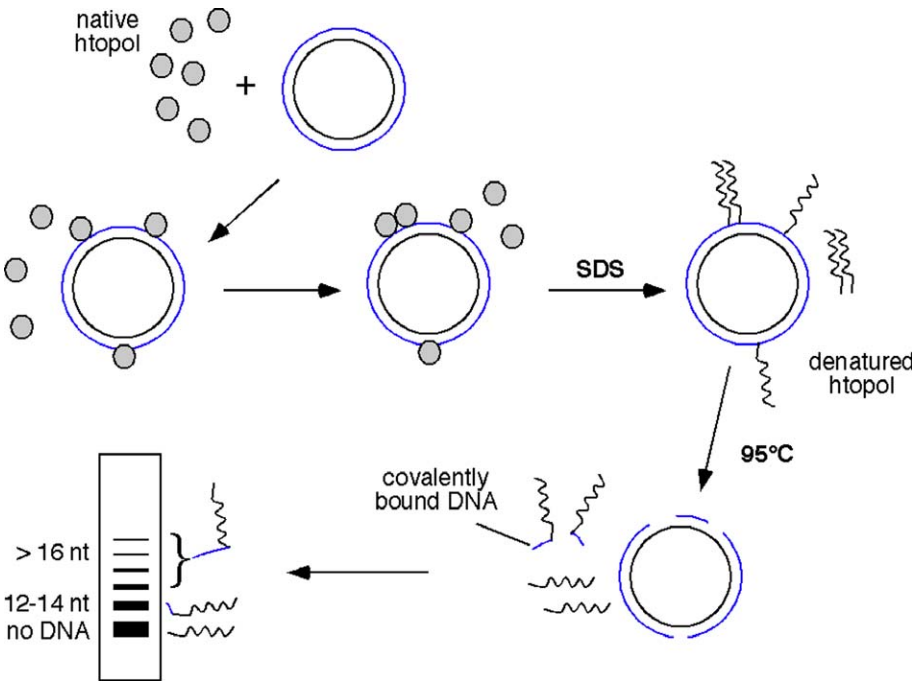

Fig. 1. Assay for the detection of htopoI double cleavage complex formation on plasmid DNA. See text for details.

molecules, the samples were heated to 95 °C. The melted-out strands with covalently bound htopoI were probed by Western-blot analysis. Melting-out of short pieces of single-stranded DNA (ssDNA) is only possible if a double cleavage reaction by two adjacent htopoI molecules has previously taken place. It should be noted that heating the sample to 95 °C should also melt-out longer strands that possibly might have been generated by two separated topoisomerase molecules that did not interact with each other via protein–protein interactions.

HtopoI and pUC19 were mixed at a ratio of 11.6 to 1 in the absence or presence of increasing concentrations of CPT. With DMSO alone no shifted htopoI was identified, but with 0.63 μM CPT or more, numerous shifted htopoI bands were detected. The most pronounced band ran only slightly slower than full-length htopoI indicating a higher molecular weight than full-length htopoI (Fig. 2A, compare lane 2 to lanes 4–9; the most prominent band of slower migrating htopoI, which in the following is referred to as "htopoI-shift", is indicated by an arrowhead). At higher concentrations of CPT more and more htopoI bands with much higher mobilities appeared, but the htopoI-shift running closest to full-length htopoI remained the predominant band. Since the reaction mixture contained only pUC19 and purified htopoI, it is highly unlikely that these band-shifts were caused by modifications of htopoI other than the attached and melted-out oligonucleotides. However, to show that these bands represent htopoI molecules attached to DNA fragments of various lengths, 300 mM NaCl was added to the reaction mixture prior to the addition of SDS. High NaCl concentrations are known to dissociate htopoI from DNA [14]. As seen in Fig. 2B, the htopoI band-shifts induced by CPT were completely converted into free htopoI molecules after addition of 300 mM NaCl. This shows that the shifted htopoI bands were caused by attached DNA fragments that in turn were generated by a second cleavage event performed by another htopoI molecule on the same plasmid molecule.

To get a rough estimate of the preferred distance between these two htopoI molecules we covalently attached a deoxyoligonucleotide with a defined length (190 nts, the so-called suicide substrate L193s), to htopoI [9]. We plotted the mobility of htopoI attached to L193s (Fig. 3A) on a semi-logarithmic graph (Fig. 3B). From such a diagram it was estimated that one attached deoxynucleotide caused a shift of the htopoI molecule that corresponded to an apparent increase of the molecular mass of about 567 Da (Fig. 3B, table). We then included the migration distances of the htopoI-shift band and that of the free htopoI in the obtained linear regression (Fig. 3B) and estimated that the attached DNA fragment consisted of about 16 ± 2 nts (Fig. 3B, table). This is very close to the previously observed distances between two htopoI molecules in a double cleavage complex with the suicide substrate, which was determined to comprise 13 nts, but also 15 or 17 nts [9].

To determine the size of the attached DNA fragment in the htopoI-shift band more precisely, we labelled the DNA attached to htopoI with ^{32}P. To get efficient labelling, we needed to increase the amount of topoI molecules attached to the desired DNA fragment. To ensure that the addition of further htopoI molecules to the reaction mixture did not change the result, we performed a titration as shown in Fig. 3C. In the previous experiments a htopoI to plasmid ratio of 11.6:1 (Figs. 2A and B) was used, but for radioac-

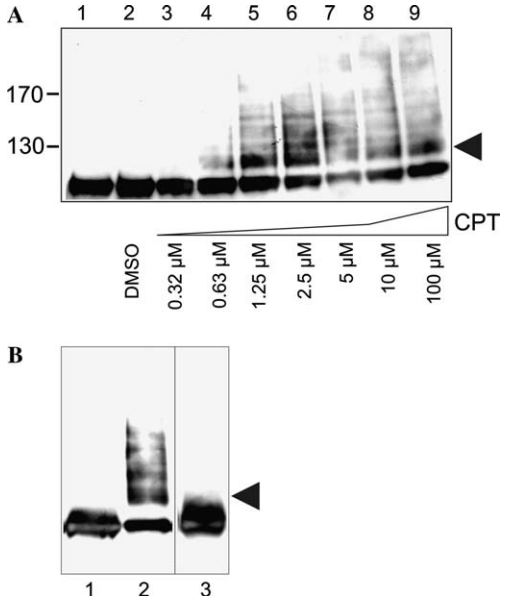

Fig. 2. HtopoI double cleavage complexes do readily form during relaxation of supercoiled plasmid DNA. 7.5% SDS–PAGE and Western blot of htopoI. (A) In a reaction volume of 20 µl, 5 pmol htopoI was incubated with 430 fmol purified pUC19 for 10 min at 37 °C. Subsequently, the indicated final concentrations of CPT (or an equivalent volume of DMSO) were added and the reaction mixtures were incubated for another 30 s at 37 °C. The reactions were finally stopped by the addition of loading buffer (including SDS) and analyzed on a 7.5% SDS–PAGE followed by Western blotting. Lane 1, 5 pmol htopoI; lanes 2–9, 5 pmol htopoI and 430 fmol pUC19 plus the indicated CPT concentrations. (B) 10 pmol htopoI was incubated with 820 fmol pUC19 for 5 min at 37 °C in a total reaction volume of 40 µl followed by an incubation with 1.25 µM CPT for 30 s. One half of the reaction volume was stopped right away as described in (A), whereas the remaining half was supplied with a final concentration of 300 mM NaCl and incubated for another 2 min at 37 °C before stopping as described above. Lane 1, htopoI; lane 2, htopoI, pUC19 and CPT; lane 3, htopoI, pUC19, CPT and 300 mM NaCl. The arrowhead indicates the htopoI-shift band.

tive labelling of 5′-OH containing DNA strands, this was increased to 38.5:1 (Fig. 3C, lane 3). When using a higher ratio of htopoI to DNA the htopoI-shift band had the same mobility. Hence, at a higher htopoI to pUC19 ratio double cleavage complexes formed more efficiently, whereas the cleavage complex pattern was not changed. Since usage of SDS would hinder the subsequent protease step, the potential double cleavage reaction was stopped by incubating at 70 °C for 10 min. Then, the protein was digested with trypsin, the DNA was precipitated, and thereafter incubated with [γ-^{32}P]ATP and T4-polynucleotide kinase. Whenever DNA is cleaved by htopoI, a 5′-OH group is generated that can be phosphorylated by a 5′ DNA-kinase and subsequently analyzed on a denaturing polyacrylamide gel. Fig. 3D shows that in the presence of CPT two distinct bands occurred that had lengths of approximately 17 and 19 nts (marked with asterisks) (lane 1). These DNA fragments were covalently attached to htopoI since without the tryptic digest no DNA fragments were observed (lane 2). The trypsin-resistant peptide still attaching to the DNA fragment has previously been deter-

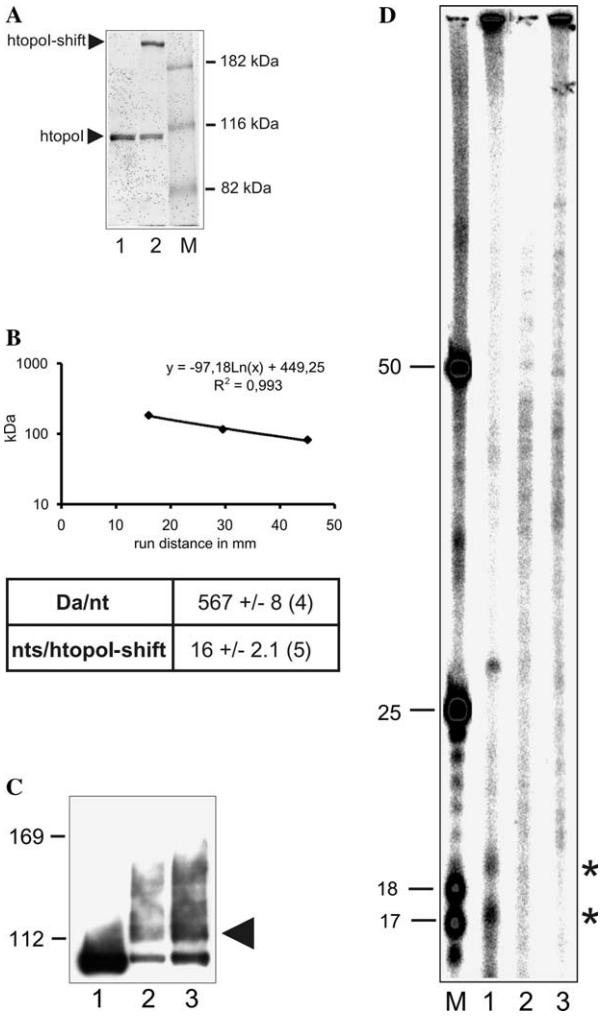

Fig. 3. The htopoI double cleavage complexes predominantly form within a distance of 12 and 14 nts. (A) 7.5% Coomassie-stained SDS–PAGE. 5.5 pmol recombinant htopoI was incubated with 11 pmol L193s for 10 min at 30 °C. Lane 1, htopoI; lane 2, htopoI and L193s. (B) Example of a semi-logarithmic graph displaying the mobility of the marker bands in (A). Based on the fitted curve an equation was obtained. Knowing that 190 nts are covalently attached to the shifted htopoI band in lane 2 of (A) we calculated the average mobility shift caused by one deoxynucleotide. The result is shown below as an average of four independent measurements (Da/nt, ± standard deviation). The mobility of the htopoI-shift band in a 7.5% SDS–PAGE was determined from five independent experiments by the use of a semi-logarithmic graph of the marker mobility. In this way we estimated the length of the deoxyoligonucleotide attached to the htopoI-shift band, which is shown in the lower part of the table (nts/htopoI-shift, ± SD). (C) Various amounts of htopoI were incubated with 143 fmol pUC19 for 8 min at 37 °C, followed by a 30 s incubation with 1.25 µM CPT and analysis by 7.5% SDS–PAGE and Western blotting as described in Fig. 2A. Lane 1, 5.5 pmol htopoI; lanes 2 and 3, 143 fmol pUC19 and 2.7 or 5.5 pmol htopoI, respectively. The arrowhead indicates the htopoI-shift band. (D) Autoradiograph of a 14% denaturing polyacrylamide gel. 5.5 pmol htopoI was incubated with 143 fmol pUC19 for 8 min at 37 °C followed by 30 s incubation with 1.25 µM CPT. The reaction was stopped by incubating for 10 min at 70 °C. The reaction mixtures were or were not treated with trypsin and then the free 5′-OH groups of the DNA were labelled with ^{32}P as described in Materials and methods. Lane M, marker; lane 1, htopoI, pUC19, CPT and trypsin treatment; lane 2, htopoI, pUC19, CPT and no trypsin treatment; lane 3, control containing only pUC19. Please note that because of the length of the DNA some undenatured material remained in the slots.

mined to comprise seven amino acids, which in turn caused a mobility shift corresponding to five apparent nts on a 14% denaturing acrylamide gel [15,16]. Thus, the corrected lengths of the DNA fragments were 12 and 14 nts, respectively. Considering the calculated length of the DNA fragment of about 16 ± 2 nts from the gel-shift experiment, it is evident that the determined 12- and 14-mer oligonucleotides were responsible for the observed htopoI-shift seen in Fig. 2. It should be noted that a random cleavage should generate fragments of about 140 nts under the experimental conditions of Fig. 3D and under the conditions Figs. 2A and B of about 447 nts, considering that htopoI can cleave on both strands of the 2686 bp plasmid. Thus, the observed cleavages at the distances of 12 or 14 nts indicate a preference for double cleavages by two adjacently bound htopoI molecules over random cleavages. In agreement with this result, atomic force microscopy of htopoI cleavage complexes induced by CPT on pUC19 revealed large htopoI structures that according to their size were interpreted to contain two closely associated htopoI cleavage complexes [17]. HtopoI double cleavage complexes were not formed due to a trapping mechanism (as that used with the suicide substrate and a covalently bound htopoI as a bait [9]) since complex formation was readily reversed by the addition of 0.3 M NaCl. Nevertheless, the here observed distance of 12 or 14 nts indicates a mechanism of complex formation very similar to that with a trapped htopoI cleavage complex, where the distance was found to be 13 nts [9].

To get a deeper insight into this mechanism, the kinetics of complex formation on plasmid DNA was investigated. An optimum of double cleavage complex formation was reached 5 min after addition of htopoI (Figs. 4A, lane 4 and B), thereafter a slow reduction was observed. After 1 min beginning complex formation was visible (Fig. 4A, lane 2), but at this time point all supercoils were already released (Fig. 4C, lane 2). During the catalytic relaxation of helical tension the structure of htopoI constantly changes whereas, when it is ''resting'', it has a confined folding [7,18,19]. Since double cleavage complexes were formed after DNA relaxation, htopoI must have been in the ''resting''

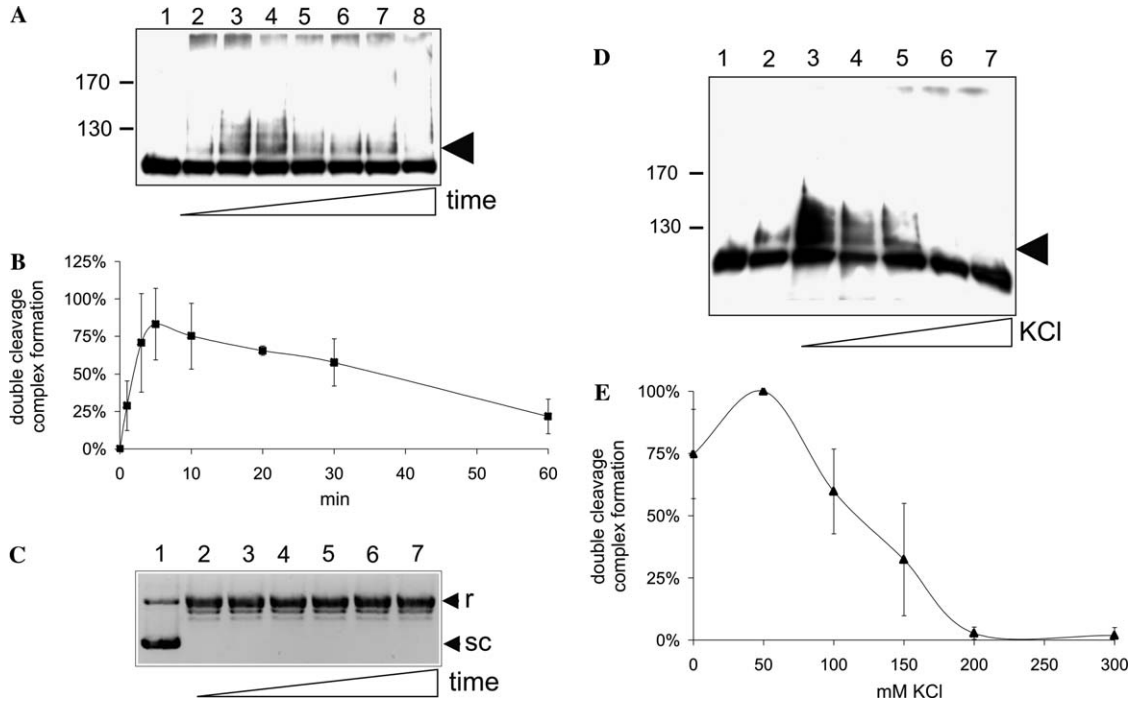

Fig. 4. HtopoI double cleavage complexes form after relaxation of superhelical tension and most efficiently at 50 mM KCl. (A) 7.5% SDS–PAGE and Western blot of htopoI. HtopoI, 5 pmol, was incubated with 429 fmol pUC19 in a total reaction volume of 20 μl for various times at 37 °C followed by 30 s incubation with 1.25 μM CPT and stopped and analyzed as described in Fig. 2A. Lane 1, pUC19; lanes 2–8, htopoI and pUC19 stopped after 1, 3, 5, 10, 20, 30, and 60 min, respectively. (B) Graphic representation of the kinetics of htopoI double cleavage complex formation as shown in (A). The SD was calculated from three independent experiments. Each experiment was quantified and the time-point showing the highest double cleavage complex formation was set to 100%. (C) 0.8% agarose gel stained with 0.6 μg/ml ethidium bromide. 70 pmol htopoI, was incubated with 6 pmol pUC19 in a total reaction volume of 140 μl at 37 °C. At each time-point 20 μl was withdrawn and treated as described in Materials and methods. Lane 1, pUC19; lanes 2 to 7, htopoI and pUC19 stopped after 1, 3, 5, 10, 20 and 30 min, respectively. (D) 7.5% SDS–PAGE and Western blot of htopoI as in (A), but incubated for 5 min at 37 °C prior to the addition of CPT in the presence of varying concentrations of KCl. Lane 1, htopoI; lanes 2–7, htopoI and pUC19 in the presence of 0, 50, 100, 150, 200, and 300 mM KCl, respectively. (E) Graphic representation of the KCl sensitivity of the htopoI double cleavage complex formation as shown in (D). The SD was calculated from three independent experiments. Each experiment was quantified and the time-point showing the highest double cleavage complex formation was set to 100%. The arrowhead indicates the htopoI-shift band. r, relaxed plasmid; sc, supercoiled plasmid.

conformation. "Resting" htopoI may dwell longer at one site and this in turn may represent a recognition signal for another htopoI.

The optimal NaCl or KCl concentrations for the relaxation of supercoiled plasmid by htopoI is 150 mM [11,14], most likely because the rate limiting factor is the release of the htopoI molecule from a relaxed plasmid to enable relaxation of the next one. Thus, a stronger DNA-binding lowers the overall relaxation rate of plasmid DNA. Na^+ and K^+ ions are thought to neutralize the negative charge of the DNA backbone and thereby reduce DNA binding of htopoI [6,14]. Since the results shown in Figs. 4A–C indicate that a resting DNA-bound htopoI may attract an additional htopoI molecule, we wanted to determine the optimal KCl concentration for complex formation. This concentration was found to be 50 mM (Figs. 4D, lane 3 and Fig. 4E), where the double cleavage complex efficiency was approximately 3-fold higher than at 150 mM KCl concentrations (Fig. 4E) indicating that a stronger DNA binding (compared to that of the relaxation reaction) is necessary for double cleavage complex formation, while the complete absence of KCl is not optimal. These findings suggest the following mechanism: a htopoI molecule actively involved in relaxing superhelical tension does not form double cleavage complexes, but when all tension has been released the enzyme seems to "rest" on DNA forming a particular conformation. This may be recognized by another htopoI molecule resulting in double cleavage complex formation. The fact that one htopoI molecule has to "find" the other one may also explain why the presence of 50 mM KCl is better than no salt, since a movement of one htopoI molecule to the other requires its release from DNA and/or sliding along the helix.

In the previous experiments we have used recombinant protein. Therefore it could be argued that double cleavage complex formation has only taken place because the recombinant enzyme was unusually modified compared to htopoI isolated from human cells. It was also possible that recombinant htopoI stored at high concentrations might have a tendency for aggregation and therefore non-physiological dimer formation. To address these points, we partially purified htopoI from growing HeLa S3 cells (Fig. 5A). After the final heparin Sepharose column htopo I was considerably enriched (Fig. 5B, lane 3). No enzyme was found in the run-through or wash-steps (data not shown) ensuring that there was no loss of a potential sub-population of htopoI during purification. Such a natural isolate of htopoI also displayed a shifted htopoI band (Fig. 5B, lane 3) as seen before with the recombinant enzyme. To determine whether this altered migration behavior was caused by the covalent attachment of a short DNA fragment, as generated during htopoI double cleavage complex formation (Figs. 2–4), we incubated the protein fraction containing HeLa cell-expressed htopoI with plasmid DNA and CPT (Fig. 5C). First of all it can be seen that htopoI from

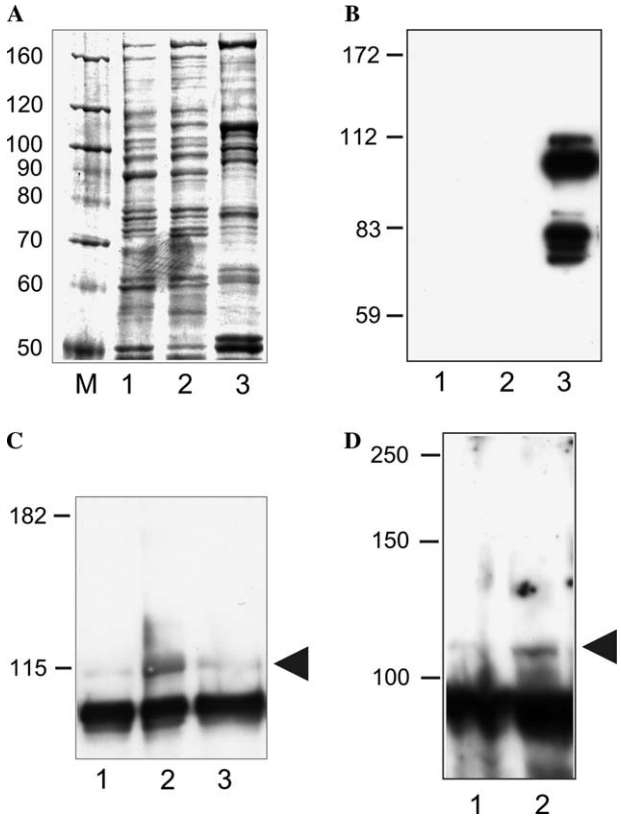

Fig. 5. HtopoI from HeLa S3 cells forms double cleavage complexes *in vitro* and possibly *in vivo*. 7.5% SDS–PAGE and Western blot of htopoI. (A) The fractions of HeLa S3 lysate were obtained as described in Materials and methods. Lanes 1–3, 1.3 μg total protein of crude extract, run-through of the phenyl Sepharose column and eluate of the heparin column, respectively. (B) The reaction was performed as described in Fig. 2A. Lane 1, 2.7 pmol htopoI; lane 2, 5.5 pmol htopoI, 143 fmol pUC19 and CPT; lane 3, 1.3 μg total protein eluate of the heparin column. (C) 2.6 μg total protein from the eluate of the heparin column was incubated with 143 pmol pUC19, CPT, and 300 mM NaCl as described in Fig. 2B. Lane 1, 2.6 μg total protein; lane 2, 2.6 μg total protein, pUC19, and CPT; lane 3, 2.6 μg total protein, pUC19, CPT, and 300 mM NaCl. (D) 7.5% SDS–PAGE and Western blot of htopoI. Cultured HeLa cells were or were not treated with 10 μM CPT for 30 min. The cells were lysed and analysed by SDS–PAGE and Western blotting. Lane 1, 3×10^5 cells; lane 2, 3×10^5 cells treated with 10 μM CPT for 30 min. The arrowhead indicates the htopoI-shift band.

HeLa cells, when incubated with plasmid and CPT, also gave rise to the attachment of a short DNA fragment (Fig. 5C, compare lanes 1 and 2) as already shown with recombinant htopoI. Complex formation was mostly reverted by the addition of 300 mM NaCl (lane 3). Second, the shifted htopoI band had exactly the same mobility as the shifted band of the recombinant htopoI (compare, e.g., Figs. 4A and 5C). Taken together, these data show that HeLa cell htopoI, in the presence of numerous other cellular proteins, was able to support double cleavage complex formation on plasmid DNA *in vitro*. This made it highly unlikely that double cleavage complex formation by recombinant htopoI represented an artefact generated during purification or storage. This also

suggested that double cleavage complexes occur in living HeLa cells as well.

To get further evidence for such an *in vivo* reaction, we tried to enhance the suspected intracellular htopoI response by treating HeLa cells with CPT for 30 min (Fig. 5D). Untreated HeLa cells displayed the same htopoI-shift band as that seen in Fig. 5C, which may indicate that double cleavage complexes also occur *in vivo*. Moreover, after incubation with CPT, the intensity of this band was markedly increased. Since CPT substantially traps double cleavage complexes [9] and because there was a strong increase in the intensity of the htopoI-shift band after CPT application, we conclude that double cleavage complexes can also be generated in living cells.

That a resting htopoI molecule bound to DNA seems to be a prerequisite for double cleavage complex formation on plasmid DNA parallels the situation during the so-called "htopoI damage response". After applying cellular stress or DNA damage, htopoI cleavage complexes have been shown to accumulate at very high levels [2,20–22] giving rise to the htopoI damage response. Since the damage response-induced cleavage complexes could be cross-linked with each other [23] it was concluded that they consist of closely associated htopoI molecules sitting side by side giving further credence to the view that htopoI double cleavage complexes may exist *in vivo*. Moreover, recent work suggested that htopoI double cleavage complexes may have been responsible for genomic translocations that are a hallmark of acute lymphoblastic leukaemia [5,24,25]. These translocations may have been caused by a recombination reaction called topoisomerase-I induced recombination repair (TIRR) that depends on the formation of htopoI double cleavage complexes *in vivo*, which until now, however, was only characterized *in vitro* [10,11]. Thus, this study and previous reports provide the first evidence for the existence of htopoI double cleavage complexes on natural DNA *in vitro* and possibly also *in vivo*. This is a novel feature of htopoI, which might contribute to a better understanding of its mechanistic properties. This in turn might be important for considering or interpreting data on the various functions of htopoI in the DNA metabolism.

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