The N-terminal Domain Anchors Human Topoisomerase I at Fibrillar Centers of Nucleoli and Nucleolar Organizer Regions of Mitotic Chromosomes*  

Received for publication, May 14, 2002, and in revised form, July 9, 2002  
Published, JBC Papers in Press, July 15, 2002, DOI 10.1074/jbc.M204738200

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DNA topoisomerase I releases torsion stress created by DNA transcription. In principle, this activity is required in the nucleoplasm for mRNA synthesis and in the nucleoli for rRNA synthesis. Yet, topoisomerase I is mostly a nucleolar protein. Current belief holds that this preference is triggered by the N-terminal domain of the enzyme, which constitutes a nucleolar import signal. Contradicting this view, we show here that nucleolar accumulation of various fragments of topoisomerase I is correlated with their lesser mobility in this compartment and not with the N-terminal domain being intact or present. Therefore, the N-terminal domain is not likely a nucleolar import signal. We show that it rather serves as an adaptor that anchors a subpopulation of topoisomerase I at fibrillar centers of nucleoli and nucleolar organizer regions of mitotic chromosomes. Thus, it provides a steady association of topoisomerase I with the rDNA and with RNA polymerase I, which is maintained in a living cell during the entire cell cycle.

DNA topoisomerase I changes the pitch of DNA helices by cutting one DNA strand and allowing rotation of the other (1). One important role for this mechanism is the release of torsion stress created by DNA transcription. Thus, topoisomerase I activity should in principle be required in the nucleoplasm for the synthesis of mRNA, and in the nucleolus for the synthesis of rRNA. However, the latter task seems to dominate. During interphase, most of topoisomerase I is located in the nucleoli and not in the nucleoplasm, although the enzyme interchanges constantly between these two compartments (2). It has been proposed that nucleolar accumulation of topoisomerase I reflects engagement in rDNA-transcription because the rRNA genes are by far the most heavily transcribed genes in the cell. In keeping with this, studies of genomic DNA cleavage by topoisomerase I have invariantly come up with sites in the rDNA (3–5). However, catalytic interactions with rDNA cannot per se account for the accumulation of topoisomerase I in the nucleoli because stabilization of covalent topoisomerase I-DNA intermediates by camptothecin immobilizes the enzyme preferentially at nucleoplasmatic sites and only to a much lesser extent in the nucleoli (2). Along this reasoning the question arises of what restricts topoisomerase I in the nucleolus and how is this phenomenon related to rDNA processing. We show here that in a living cell a sub population of topoisomerase I is constantly anchored at the fibrillar centers of the nucleolus or the nucleolar organizer region of mitotic chromosomes and that this is due to an adaptor function of the N-terminal domain of the enzyme.

EXPERIMENTAL PROCEDURES

Cloning and Cell Culture—GFP1 chimera of human topoisomerase I and of various fragments of it (see Fig. 3A) were stably expressed in the human embryonal kidney cell line 293 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) using the bicistronic expression vector pMC-2P (6) in which the translational initiation of the selection marker puromycin-N-acetyl transferase in the second cistron is mediated by an internal ribosome entry site from poliovirus. The coding sequence for GFP was inserted (HindIII/EcoRI) in front of the internal ribosome entry site by linker PCR using pEGFP (CLONTECH) as a template. Unless GFP alone was to be expressed, the stop codon of GFP was replaced with MinI/Apal restriction sites to allow for in-frame fusions to PCR-generated coding sequences of human topoisomerase I, topoisomerase I(Phe723), or various fragments of human topoisomerase I. Whenever such fragments were lacking the endogenous nuclear localization signal sequences of topoisomerase I (i.e. TI175–765, TI190–765, TI210–765, and TI1–133 (see Fig. 3A)), cloning was done with PCR-primers supplemented with the nuclear localization signal of SV40. Cells were grown in Dulbecco’s modified Eagle’s medium with Glutamax-I (Invitrogen) and transfected using LipofectAMINE (Invitrogen). Stably transfected cell lines were selected after 2 days with 0.55 μg/ml of puromycin and maintained under selection.

Characterization of Fusion Protein Expression—Cells expressing the various GFP chima of a constitutive manner were harvested, resuspended in PBS, and lysed by addition of an equal volume of 2-fold lysis buffer (25 mM Tris-HCl, pH 6.8, 10% SDS, 8 mM urea, 20 mM glycerol, 0.04% bromophenol blue, 10 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml of aprotinin, and 10 μg/ml of peptatin A. Lysates equivalent to 5 × 106 cells were applied to each slot of an SDS-polyacrylamide gel. After electrophoresis proteins were electroblotted onto polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, MA). The membranes were subsequently blocked with 2% bovine serum albumin, 0.05% Tween 20 in PBS (167 mM NaCl, 25.5 mM Na2HPO4, 2 mM KH2PO4, pH 7.5) and then incubated for 1 h in the same buffer with rabbit peptide antibodies against the last 18 C-terminal amino acid residues of human topoisomerase I (Genosys, Cambridge, UK) or with mouse monoclonal GFP antibodies (CLONTECH, Heidelberg, Germany). After washing, the filters were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies diluted in PBS containing 2% bovine serum albumin and 0.1% Tween 20. Following extensive washing with the same buffer, labeled protein bands were finally visualized with the ECL Plus system (Amersham Biosciences). Fig. 1 summarizes representative results. GFP chimera of full-length topoisomerase I, topoisomerase I(Phe723), and the various N-terminally truncated fragments were separated in 8% gels. Western blots of these constructs

* This work was supported by the Deutsche Forschungsgemeinschaft (Bo 910/3-1, Bo 910/4-1, GRK 639, and HA 1434/13-1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: GFP, green fluorescent protein; PBS, phosphate-buffered saline; FRAP, fluorescence recovery after photobleaching.
w we used a Zeiss LSM 510 inverted confocal laser scanning microscope. Both microscopes were equipped with a heated cell chamber (Bioptechs Inc., Butler, Pennsylvania) and a heated 63×1.4 NA oil immersion objective, allowing culturing of the cells under the microscope at 37 °C. For FRAP measurements, fluorescent images of a single optical section were taken at 1.8-s time intervals before (n = 5) and after bleaching of a circular area of 20 μm nominal laser power width with three iterations. Imaging scans were acquired with the laser power attenuated to 0.1–1% of the bleaching intensity. For quantitative analysis of FRAP, fluorescence intensities of the bleached region and of the entire cell nucleus were measured at each time point. Data were corrected for extracellular background intensity and for the overall loss in total intensity resulting from the bleach pulse itself and from the imaging scans. The relative intensity of the bleached area ($I_{rel}$) was calculated according to Ref. 7.

The Effects of Temperature and Mounting—It should be emphasized that all microscopic data presented here were obtained with cells living under the microscope at 37 °C. Morphology and mobility of GFP-topoisomerase I was different when observed at 20 °C or when native cell specimen were mounted in 50% glycerol as done in previous studies (8–12). This is demonstrated in Fig. 2. At 37 °C GFP-topoisomerase I exhibited a granular pattern in the nucleoli and a homogenous one of much lesser intensity in the nucleoplasm (Fig. 2A, top row). Upon transfer of the cells to ambient temperature, the granular pattern in the nucleoli was progressively lost over time and replaced with a homogenous one, whereas the nucleoplasmatic pattern became inhomogenous and, moreover, interspersed with enzyme aggregates that were not apparent at 37 °C (Fig. 2A, compare rows 2–4 with row 1). Upon mounting the cells in 50% glycerol, GFP-topoisomerase I became redistributed from the nucleoli to the nucleoplasm to such an extent that it appeared evenly distributed between the two compartments (Fig. 1A, row 5) or even excluded from the nucleoli (Fig. 2A, row 6). Fig. 2B demonstrates the effect of lowering the temperature on the mobility of GFP-topoisomerase I. FRAP kinetics in the nucleoplasm (top) was clearly slower at ambient temperature than at 37 °C (compare circles with triangles). This effect was even more pronounced in mitotic chromosomes where FRAP was rapid and complete at 37 °C, whereas it was much slower and incomplete at ambient temperature (Fig. 2B, bottom, compare circles and triangles). Thus, topoisomerase I seems not to conform to the widely accepted working hypothesis that the mobility of nuclear proteins is the same at ambient temperature and at 37 °C (7). Therefore, it seemed advisable to study this protein at 37 °C and to control this experimental condition in a stringent manner.

Immunohistochemistry—For immunostaining of RNA polymerase I, fibrillarin, and nucleolin, cells were grown on microscopic glass slides, permeabilized (0.07% Triton X-100 in PBS for 30 s at 37 °C), and then fixed with PBS containing 2% paraformaldehyde (15 min, 4 °C). The following steps were carried out at ambient temperature. After washing with PBS, the cell samples were blocked for 1 h with PBS supplemented with 2% bovine serum albumin and 5% goat serum. Then they were incubated for 1 h with the primary antibodies diluted in PBS. Human autoimmune serum S18 (13) was used at a 1:800 dilution to detect RNA polymerase I. Human autoimmune serum S18 (13) was used at a 1:800 dilution to detect RNA polymerase I. Human autoimmune serum S18 (13) was used at a 1:800 dilution to detect RNA polymerase I. Human autoimmune serum S18 (13) was used at a 1:800 dilution to detect RNA polymerase I. 

RESULTS

Nucleolar Accumulation of Topoisomerase I Is Governed by Protein Mobility—Fig. 3A shows representative images of the in vivo localization of GFP chimera of human topoisomerase I or of various fragments of it, and of GFP alone. Upon comparison of green fluorescent images (middle column) with corresponding images obtained by transmitted light (left column), it is readily apparent that all of these proteins were expressed in the nucleolus. With the exception of GFP-topoisomerase I, GFP alone, they all accumulated in the nucleoli. Nucleolar accumulation was clearly not correlated with the N-terminal domain being present or not; on the one hand, truncated versions having lost this portion to a varying extent (TI$^{3-765}$, TI$^{1175-765}$, TI$^{1900-765}$, and TI$^{210-765}$) showed a similar nucleolar accumulation as the entire enzyme (Topo I), the same holding true for the N-terminal domain alone (TI$^{1-210}$) and for various

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subfragments of it (TI$^{1-133}$ and TI$^{133-210}$). On the other hand, the active site mutant (TI$^{Phe725}$) did not accumulate in the nucleoli, although it still had the entire N-terminal domain.

We have previously speculated that nucleolar accumulation of topoisomerase I could be simply due to its lesser mobility in this compartment, as compared with the nucleoplasm (2). To test this hypothesis, we compared here by FRAP the mobility of various topoisomerase I fragments inside and outside of the nucleolus. A representative summary of these data shown in Fig. 3B demonstrates that those enzyme fragments having the capability of accumulating inside the nucleoli (TI, TI$^{133-765}$, and TI$^{210-765}$), also had a slower fluorescence recovery in this compartment than in the nucleoplasm. In contrast, the active site mutant (TI$^{Phe725}$), incapable of accumulating inside the nucleoli, had the same mobility everywhere in the nucleus. In fact, nucleolar accumulation of the active site mutant seemed to be precluded by its attenuated mobility in the nucleoplasm, rather than by alterations in the nucleolus. In summary, these data support our previous hypothesis that differences in protein mobility govern nucleolar localization of topoisomerase I (2), whereas our findings argue clearly against earlier suggestions that the capability of topoisomerase I accumulating inside the nucleoli should be a function of the N-terminal domain of the enzyme (8–10, 12) or of parts of it (15). However, nucleolar accumulation due to a decreased mobility in this compartment seems to be a feature not shared inadvertently by all proteins present in the nucleolus because GFP alone exhibited a lesser intensity in the nucleoli (Fig. 3A, bottom panel) although it is freely diffusible in all parts of the cell nucleus (7).

**FIG. 2.** Effects of temperature and embedding on localization and mobility of GFP-topoisomerase I. A, localization. Cells expressing GFP-topoisomerase I were cultured under the microscope and inspected at 37 °C (row 1), were kept at ambient temperature for the time periods indicated before inspection at ambient temperature (rows 2–4), or were mounted in 50% glycerol and then inspected at ambient temperature (rows 5–6). For each condition, representative cell images obtained by phase contrast (left) and green fluorescence (middle) are shown. The right column shows single nucleoli, indicated in corresponding images in the middle column by boxes, which were subjected to an additional 3-fold magnification and contrast enhancement. B, mobility. FRAP analyses of cells expressing GFP-topoisomerase I were carried out at 37 °C (○) or at ambient temperature (○). For both conditions a nucleoplasmatic region (top) or a section of condensed metaphase chromosomes (bottom) was bleached, and fluorescence intensities in the bleached spots were measured and plotted as the relative recovery over time after the bleach pulse (at 0 s). Bleach spots are indicated by circles in the pre-bleach images. Mean values from six individual cells and three independent experiments are shown. Standard deviations were in each case less than 5% of the mean values (not displayed). Representative images of the cells are shown above the curves.
observations suggest that the N-terminal domain per se anchors topoisomerase I at a distinct substructure of the nucleolus. This process does not require the enzyme to be capable of DNA transesterification and formation of covalent DNA complexes, and is not brought about by enzyme activity per se. Thus, it is entirely independent of the catalytic activity of
topoisomerase I and solely depends on the N-terminal domain.

To get an idea at which nucleolar substructure topoisomerase I is anchored, we counterstained cells expressing GFP-topoisomerase I with antibodies directed against marker proteins of various nucleolar subcompartments. RNA polymerase I (Fig. 3D, bottom), which delineates the fibrillar centers (16), co-localized entirely with GFP-topoisomerase I. Antibodies against fibrillarin (Fig. 3D, middle), which delineate the dense fibrillar component surrounding the fibrillar centers (17), appeared to stain a larger structure that was more diffuse around the edges and covered a wider area than that occupied by topoisomerase I. Antibodies against nucleolin delineating the granular component and other peripheral parts of the nucleolus (18) stained surrounding structures and were not co-localized with topoisomerase I. Thus we concluded that the nucleolar substructure, where topoisomerase I is anchored, must be the fibrillar centers of the nucleolus. The data in Fig. 3A demonstrate that the entire non-conserved N-terminal domain of the enzyme (amino acid residues 1–210) is required for this function because this was the only fragment exhibiting the same granular pattern in the nucleoli as the entire enzyme. It should also be kept in mind that accumulation of topoisomerase I at the fibrillar centers of the nucleolus is rapidly lost at ambient temperature (Fig. 2A) and thus seems to require cells to be alive. In contradiction to previous reports (15), the lack of co-localization with nucleolin shown in Fig. 3D (top) argues against a role of this protein in the nucleolar targeting of topoisomerase I.

During Mitosis, the N Terminus Anchors Topoisomerase I at Nucleolar Organizer Regions—Recently, we have observed that during mitosis topoisomerase I is more or less completely bound to the chromosomes (2). In the light of the findings presented in the previous paragraph, we were curious to see whether the N-terminal domain would also play a role in this allocation. Prima facie, this seemed not to be the case because a fragment lacking the entire N-terminal domain showed the same chromosomal pattern as full-length topoisomerase I, whereas a fragment comprising just the N-terminal domain seemed to be distributed equally between the mitotic cytoplasm and the chromosomes (Fig. 4A, bottom, compare left, middle, and right). However, confocal sectioning revealed that the N-terminal fragment highlighted distinct dot-like structures within the metaphase plate (Fig. 4B, arrows). When following such structures through a mitotic cycle (Fig. 4C), we found that they were paired at metaphase (0°) and that each pair was split at anaphase (12°–17°), giving rise to individual daughter structures that were maintained during telophase (19°–25°), whereas toward G1-phase (31°–45°) they moved into closer proximity of each other (31°) and finally merged (36°). Eventually, all of them ended up in a reforming nucleolus (45°, arrowheads).

These observations suggested that the N-terminal domain of topoisomerase I binds to certain chromatin regions, thereby possibly defining a chromosomal subpopulation normally not apparent on the background of enzyme bound to the entire length of the chromosomes. Assuming that this subpopulation might again be more tightly bound than the rest of the enzyme, we subjected mitotic cells to the same detergent extraction as described in Fig. 3C. For the full-length enzyme (Fig. 4D, top), this procedure indeed unveiled similar dots as delineated by the N-terminal domain alone (Fig. 4C), whereas with the C-terminal fragment (Fig. 4D, bottom) this was not the case. Here, most of the protein was extracted from the chromosomes within 4 min after adding the detergent, without revealing any dot-like structures. It should be noted that the dots delineated by the N-terminal domain alone were also resistant to extraction (not shown). Thus, the N-terminal domain seems to anchor topoisomerase I at certain regions of mitotic chromosomes, and, considering the fate of these structures at the end of mitosis (Fig. 4C, 45°), they must be the same, similar, or related to
those where the enzyme is anchored during interphase. The most likely candidates are the nucleolar organizer regions of the acrocentric chromosomes (19), which contain the major rDNA genes, are located in the fibrillar centers of the nucleolus during interphase, and are normally associated with the machinery of rRNA transcription (16, 20). We confirmed this assumption by counterstaining mitotic cells expressing GFP-topoisomerase I with antibodies against RNA polymerase I. Upon comparing the GFP signal of topoisomerase I (middle) with the immunofluorescent signal specific for RNA polymerase I (right), it becomes apparent that both proteins co-localize at globular structures within the mitotic chromatin and that these are the only distinct structures delineated by the two proteins. A similar co-localization with RNA polymerase I could also be demonstrated for the N-terminal domain of topoisomerase I alone (not shown).

Summing up all the data, it appears that the N-terminal domain anchors topoisomerase I at the nucleolar organizer regions and related structures in the fibrillar centers of interphase nucleoli. This anchoring is clearly a specific feature of living cells, and the mechanism is dedicated to keeping a certain fraction of topoisomerase I in close proximity to the rDNA and the machinery of rRNA transcription throughout the entire cell cycle.

**DISCUSSION**

Immunohistochemical studies have firmly established topoisomerase I as a mostly nucleolar protein (5, 21–23), but it is unclear what actually directs the enzyme to the nucleolus and for what purpose. The most likely candidate responsible for targeting topoisomerase I to defined locations within the cell nucleus is the non-conserved N-terminal domain spanning amino acid residues 1–210 of the human enzyme. This domain distinguishes eukaryotic topoisomerase I from a minimal variant encoded by vaccinia virus (24) and from other microbial recombinases more distantly related (25). It contributes scarcely to the activity of the enzyme in vitro (26), but it is believed to determine the biological properties of topoisomerase I. Most notably, it seems to be a docking place for interacting proteins, such as nucleolin (27). Current belief holds that it also serves as a signal sequence triggering the nucleolar import of topoisomerase I (9, 12), with nucleolin interactions possibly driving the process (15).

However, we have recently observed that in a living cell topoisomerase I exchanges between nucleoli and the nucleoplasm in a rapid and continuous manner, suggesting that the distribution of the enzyme between these compartments is more likely governed by differences in residence time than by directing signals (2). In keeping with this, we show here that nucleolar accumulation of various truncated versions of topoisomerase I is correlated with their lesser mobility in this compartment and not with the N-terminal portion being intact or present. Thus, the N-terminal domain cannot be a trigger for the import of topoisomerase I into the nucleolus.

Alternatively, we would like to propose that the N-terminal domain anchors topoisomerase I at distinct locations within the nucleolus. We show here that in a living cell topoisomerase I does not accumulate in the nucleolus in an indistinct manner. It rather concentrates at certain substructures, which we identify as the fibrillar centers. This property is clearly retained by the active site mutant and by a fragment comprising the entire N-terminal domain (amino acids 1–210), whereas fragments lacking portions of this domain accumulate in the nucleolus in an amorphous and unstructured manner. Nucleolin has previously been suggested to direct topoisomerase I to the nucleolus (15), but it is unlikely that it plays such a role in the targeting mechanism described here. First, it does not co-localize with topoisomerase I at the subnucleolar level. Second, fragments of topoisomerase I lacking most of the N-terminal domain but retaining the nucleolin-binding site (27) do not show a distinct accumulation at the fibrillar centers characteristic of the entire enzyme.

In summary, our observations suggest that the N-terminal domain serves as an anchor, which increases the local concentration of topoisomerase I at the fibrillar centers of the nucleolus. Interestingly, this domain plays a similar role at mitosis, where it associates topoisomerase I with the nucleolar organizing regions of mitotic chromosomes, a finding previously also indicated by immunohistochemistry (28, 29). At both locations (fibrillar centers of the nucleolus and nucleolar organizing regions) topoisomerase I co-localizes with RNA polymerase I, which is believed to form a holo-enzyme together with a variety of other proteins also involved in rRNA transcription (30). Our data suggest that topoisomerase I could be a regular component of this holo-enzyme. This suggestion is made even more plausible by the long-standing observation that topoisomerase I co-purifies with RNA polymerase I (31). One is tempted to speculate that in this way a subfraction of topoisomerase I remains steadily associated with the rDNA and with the machinery required for RNA transcription, which is also attached to the nucleolar organizing regions (16, 20).

However, the precise localization of rRNA transcription within the nucleolus is still open to discussion. Some authors suggest that it is confined to the fibrillar centers (32). Others claim that these just serve as a storage place for inactive RNA polymerase I and other transcription factors, whereas transcription occurs largely at the boundary to the dense fibrillar component (33). This controversy provides us with two alternative interpretations of our data. One is that topoisomerase I at the fibrillar centers is a storage form. The other is that it is heavily involved in the relaxation of super-coils generated during transcription of rDNA. The second model is supported by the finding that the N-terminal domain targets topoisomerase I to sites of transcriptional activity (34) and by the observation that ongoing transcription seems to recruit topoisomerase I to the nucleoli (12). The inverse should be the case, if the enzyme was stored there in an inactive form. The second model is also more appealing from a teleological point of view. The rRNA genes are the most heavily transcribed genes in the cell, and it is making sense that a substantial portion of the machinery required for this process including topoisomerase I should always stay in place. The second model, however, somewhat disavored by our own recent observation, is that campothecin treatment immediately induces depletion of topoisomerase I from the nucleoli (2). If topoisomerase I was heavily

![Fig. 5. Topoisomerase I and RNA polymerase I co-localize at globular structures within the mitotic chromatin. Cells expressing GFP-topoisomerase I were stained with antibodies against RNA polymerase I and visualized by confocal microscopy (same as in Fig. 3D). A representative cell at mitosis is shown by transmitted light on the left. The box indicates the area for which corresponding confocal mid-plane images of green GFP-fluorescence (middle) and immunofluorescence specific for RNA polymerase I (right) are shown at a 2-fold higher magnification.](image-url)
engaged in rDNA catalysis at the fibrillar centers, then it should be fixed there by camptothecin and not be depleted from the nucleoli. However, we do not know if the nucleoli are as accessible to camptothecin as the nucleoplasm.

Acknowledgments—We are grateful to Jörg Hacker and Hilde Merkert, Research Center for Infectious Diseases, University of Würzburg, Germany for generously providing access to a confocal laser scanning microscope. The clone of topoisomerase P*7273 was a generous gift of James J. Champoux, Dept. Microbiology, University of Washington, Seattle, WA. Antibodies against RNA polymerase I and fibrillarin were kindly provided by Ulrich Scheer and Robert Hock, Institute of Developmental Biology, Theodor Boveri-Institute of Biosciences, University of Würzburg, Germany.

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