Rapid exchange of mammalian topoisomerase IIα at kinetochores and chromosome arms in mitosis

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A stable cell line (GT2-LPk) derived from LLC-Pk1 was created in which endogenous DNA topoisomerase IIα (topoIIα) protein was downregulated and replaced by the expression of topoII fused with enhanced green fluorescent protein (EGFP–topoIIa). The EGFP–topoIIa faithfully mimicked the distribution of the endogenous protein in both interphase and mitosis. In early stages of mitosis, EGFP–topoIIa accumulated at kinetochores and in axial lines extending along the chromosome arms. During anaphase, EGFP–topoIIa diminished at kinetochores and increased in the cytoplasm with a portion accumulating into large circular foci that were mobile and appeared to fuse with the reforming nuclei. These cytoplasmic foci appearing at anaphase were coincident with precursor organelles of the reforming nucleolus called nucleolus-derived foci (NDF). Photobleaching of EGFP–topoIIa associated with kinetochores and chromosome arms showed that the majority of the protein rapidly exchanges (t1/2 of 16 s). Catalytic activity of topoIIα was essential for rapid dynamics, as ICRF-187, an inhibitor of topoIIα, blocked recovery after photobleaching. Although some topoIIα may be stably associated with chromosomes, these studies indicate that the majority undergoes rapid dynamic exchange. Rapid mobility of topoIIα in chromosomes may be essential to resolve strain imparted during chromosome condensation and segregation.

Introduction

DNA topoisomerase II (topoII)* is essential in proliferating cells and is a major target for many anticancer drugs (Bakshi et al., 2001; Wang et al., 2001). In mammals, two genes code for isoforms of topoisomerase II, termed α and β, and alternative splicing confers further diversity (Petruti-Mot and Earnshaw, 2000). TopoIIα has been proposed to play both catalytic and structural roles in mitotic chromosome segregation. Genetic studies in yeast and inhibitor studies in mammalian cells suggest that topoIIα is required to resolve sister chromatid catenations during separation of the chromatids (Uemura et al., 1987; Holm et al., 1989; Gorbsky, 1994). In chromosomes that have been isolated from cells arrested in M phase, the bulk of topoII is tightly associated, suggesting that topoII forms a stable structural component or scaffold of chromosome architecture (Earnshaw et al., 1985). A study of rhodamine-labeled topoII in Drosophila embryos challenged this model with evidence that much topoII diffuses from the chromosomes at progressive stages of mitosis (Swedlow et al., 1993). The stability of topoIIα protein is cell cycle regulated, high during G2/M phases, and becoming unstable in the subsequent G1 phase (Heck et al., 1988).

Some immunolocalization and electron microscopy studies show a distribution of topoII in an axial core extending the length of the chromosome (for review see Warburton and Earnshaw, 1997). However, in both Drosophila embryos and condensed chromosomes formed in vitro in Xenopus extracts, topoII shows more uniform distribution on the chromosome arms (Hirano and Mitchison, 1993; Swedlow et al., 1993). In some immunolabeling studies, the enzyme was reported to be concentrated at kinetochores (Taagepera et al., 1993; Gorbsky, 1994; Rattner et al., 1996; Sumner, 1996). In interphase mammalian cells, various groups report differences in the subnuclear distribution of topoIIα with some but not all studies noting a concentration in the nucleoli (Meyer et al., 1997; Khelifa and Beck, 1999; Mo and Beck, 1999). To investigate the dynamics of topoIIα in living cells, we produced a stable cell line expressing EGFP–topoIIα. FRAP...
experiments show that most topoIIα exchanges rapidly between cytoplasmic and kinetochore/chromosome-bound pools, and thus does not constitute a static structural chromosome scaffold. We also report a novel localization of topoIIα in late anaphase and telophase to discrete cytoplasmic foci that appear to incorporate into the reforming nucleoli during telophase.

Results and discussion

Establishment of a stable cell line expressing EGFP-topoIIα

To track the localization and dynamics of topoIIα in unperturbed living cells, we constructed a stable cell line, called GT2-LPk, that constitutively expresses EGFP–topoIIα. The overexpression of active topoII is cytotoxic, and although transient expression is possible, it has not previously been possible to obtain stable lines that constitutively express tagged forms of the enzyme (Mo et al., 1998; Mo and Beck, 1999). We compared the expression levels of endogenous topoIIα in the parental LLC-Pk and GT2-LPk lines. We found that expression of endogenous topoIIα was diminished in the GT2-LPk line, presumably accounting for the ability of the GT2-LPk line to escape cytotoxicity from topoIIα overexpression (Fig. 1 A). Immunoprecipitated EGFP–topoIIα efficiently catalyzed decatenation of kinetoplast DNA (Fig. 1 B). EGFP–topoIIα bound to protein A beads showed equivalent or greater catalytic activity compared with purified human topoIIα, a gift from Dr. Neal Osheroff (Vanderbilt University, Nashville, TN) (unpublished data).

EGFP–topoIIα localization in living cells

In living cells in interphase, EGFP-topoIIα was present diffusely in the nucleoplasm and concentrated in the nucleoli (Fig. 1 C). Nucleolar labeling persisted through early prophase when concentration of EGFP-topoIIα on chromosomes and at kinetochores became apparent. In living cells during prometaphase and metaphase topoIIα localized to thin lines running down the lengths of the chromosome arms (Fig. 1 C). Higher resolution images in fixed cells revealed that EGFP-topoIIα was concentrated along the central axis of each chromatid arm (Fig. 1 D). This distribution is consistent with the axial distribution of topoII observed in immunolabeling studies of fixed mammalian cells and isolated chromosomes (Earnshaw and Heck, 1985; Gorbsky, 1994; Rattner et al., 1996; Sumner, 1996). In anaphase, centromere concentration diminished while the lo-
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In late anaphase and telophase, cytoplasmic levels of topoIIα increased similar to the behavior of rhodamine-labeled topoII in Drosophila (Swedlow et al., 1993). The anaphase and telophase dynamics of topoIIα may also be preliminary to the decrease in topoIIα protein stability that occurs after mitosis (Heck et al., 1988). In contrast to a previous immunolabeling study (Barthelmes et al., 2000), we never observed concentration of EGFP–topoIIα at centrosomes at any stage of the cell cycle.

In late anaphase we observed a novel concentration of topoIIα in cytoplasmic foci (Figs. 1 C, telophase, and 2 A [Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200202053/DC1]). The foci were motile and appeared to move toward and fuse with the reforming nuclei in telophase. During telophase, most cytoplasmic topoIIα appeared to reenter the forming nuclei, and the concentration of EGFP–topoIIα in the nucleoli reappeared. The approximate size, behavior, and appearance of the cytoplasmic EGFP–topoIIα-containing foci were reminiscent of previously described dynamic structures termed nucleolus-derived foci (NDF) (Dundr et al., 2000). These cytoplasmic NDFs apparently travel to nuclei where they fuse with other nucleolar precursors within the nuclei, e.g., prenucleolar bodies, to reform the interphase nucleoli (Olson et al., 2000). We found that EGFP–topoIIα foci colocalized with antibody to B23, a known component of the NDFs (Fig. 2 B). In contrast, EGFP–topoIIα in anaphase and telophase GT2-LPk cells did not colocalize with nuclear pore antigens (Fig. 2 C).

**TopoIIα exchanges rapidly in mitotic chromosomes**

To quantify topoIIα exchange in living cells in mitosis, we used FRAP. If topoIIα served a static structural or nucleo-

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**Table I. Recovery of EGFP–topoIIα after photobleaching at kinetochores and chromosome arms**

<table>
<thead>
<tr>
<th>Structure</th>
<th>t1/2 s (SD)</th>
<th>Range</th>
<th>% recovery (SD)</th>
<th>Range</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetochores</td>
<td>15.7 (4.7)</td>
<td>9.0–22.4</td>
<td>61.9 (14.7)</td>
<td>42.8–86.7</td>
<td>8</td>
</tr>
<tr>
<td>Chromosome arm</td>
<td>15.5 (3.6)</td>
<td>9.9–22.5</td>
<td>75.9 (13.0)</td>
<td>52.4–90.0</td>
<td>10</td>
</tr>
</tbody>
</table>

Kinetochores and chromosome arms of GT2-LPk cells were photobleached with a nitrogen dye laser. Recovery of fluorescence was measured in the photobleached area from images taken at intervals after photobleaching. After correcting for background and loss of fluorescence due to image capture, measurements were used to calculate halftime recovery (t 1/2) and the percentage of recovery.
skeletal role in maintaining the organization of the kinetochore or chromosome arm, then recovery should be slow. We found that recovery of the majority of the EGFP–topoIIα/H9251 at kinetochores and chromosome arms occurs rapidly (Fig. 3 and Table I). Because the average recovery was 100%, there may be a subpopulation of kinetochore and chromosome-associated topoIIα that is more stably associated (for kinetochores an average of 38%; for chromosome arms an average of 24%). We found that the exchange of topoIIα occurred at all stages of mitosis and did not differ markedly from prophase through anaphase (Fig 3). Recovery of fluorescence likely reflects rapid exchange between the pools of topoIIα bound at chromosomes and kinetochores with the pool of cytoplasmic topoIIα.

The rapid exchange of the majority of topoIIα in vivo contrasts to the tight association of topoIIα in chromosomes isolated from mitotic cells. In isolated chromosomes, topoII is resistant to DNAse treatment and extraction with high salt, and has thus been proposed to function as part of a stable structural scaffold for the mitotic chromosome. Although there may be a minor population of topoII that is stably associated with chromatin, we find the majority to exchange rapidly with topoIIα in the cytoplasm. Thus, in vivo, it appears unlikely that the bulk of topoIIα serves a static nucleoskeletal role in maintaining mitotic chromosome structure.

Rapid exchange of topoIIα requires catalytic activity
To determine whether catalytic activity of topoIIα was required for the rapid exchange observed in our FRAP analyses, we used a chemical topoIIα inhibitor, ICRF-187. ICRF-187 is an inhibitor of the bisdioxopiperazine class that blocks the enzyme by trapping it in the postcleavage closed clamp form (Roca et al., 1994). Applied to cells in mitosis, this inhibitor inhibits chromosome condensation and induces massive failure of chromosome segregation at anaphase in LLC-Pk cells, the parental line of the GT2-LPk cells (Gorbsky, 1994). Similarly, we found that ICRF-187 inhibits normal chromosome condensation and chromosome segregation in GT2-LPk cells (Fig. 4, A and B). In mitotic cells treated with ICRF-187, we detected only minimal recovery of EGFP–topoIIα into the bleached areas over a 10-min period (Fig. 4 C).

To corroborate the effects of ICRF-187 on the dynamics of topoIIα in mitotic cells, we also tested its effects in interphase nuclei. Treatment of cells with ICRF-187 greatly diminished the level of EGFP–topoIIα concentrated in the nucleolus. When tested by FRAP analysis of interphase cells,
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again ICRF-187 treatment was found to cause a marked decrease in the rate and extent of fluorescence recovery when compared with nondrug-treated interphase cells (Fig. 4, C and D). Together, these results suggest that the rapid exchange noted for EGFP–topoIIα in unperturbed cells requires catalytic activity. Moreover, ICRF-187 treatment slows the exchange rate of EGFP–topoIIα, lending further support to the idea that the EGFP–topoIIα protein is functional in vivo.

Rapid exchange of chromosome-associated and cytoplasmic pools of topoII

To further test whether the rapid exchange of topoIIα was somehow restricted to the protein associated with the chromosomes or occurred by exchange with a soluble cytoplasmic pool, we used fluorescence loss in photobleaching. We repeatedly photobleached a small area in the cytoplasm (Fig. 5 A) or on one region of the chromosomes of cells treated with a microtubule inhibitor to inhibit chromosome movement (Fig. 5 B; Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200202053/DC1). Loss of fluorescence of EGFP–topoIIα from unbleached mitotic chromosomes occurred upon repetitive bleaching at the distant area (Fig. 5). These data demonstrate that topoIIα mobility is not regionally confined. TopoIIα associated with chromosomes and kinetochores is mobile and exchanges with cytoplasmic pools of the enzyme.

Another study recently appeared analyzing GFP derivatives of topoIIα and topoIIβ in stable lines of 293 cells (Christensen et al., 2002). In general, the results from these authors and ours are consistent in describing the overall localization of topoIIα in interphase and mitotic cells. Both studies also agree that most topoIIα undergoes rapid exchange with the cytoplasm, and the studies concur on the necessity for topoIIα catalytic activity in rapid exchange. The studies do differ with regard to certain conclusions regarding topoIIα in mitotic chromosomes. Whereas Christensen et al. (2002) report that nearly 100% of topoIIα turns over rapidly, our results indicate that a population of topoIIα associated with the kinetochores and chromosome...
arms turns over more slowly. Moreover, whereas Christensen et al. (2002) conclude that in living cells topoII/H9251 is uniformly distributed across the width of the chromosome arms, our evidence favors the idea that topoII/H9251 concentrated toward the axes of the arms. There are several potential sources for the differences in the two studies. Apart from inherent differences between the two parental cell lines (LLC-Pk vs. 293), the GT2-LPk derivative line used in our work has downregulated levels of endogenous topoII/H9251 that is replaced by EGFP–topoII/H9251. The 293 cell derivatives of Christensen et al. (2002) did not show evidence of downregulation of the endogenous enzyme. In addition, the photobleaching studies of mitotic chromosomes performed by Christensen et al. (2002) were carried out on cells arrested at M phase by treatment with the microtubule stabilizer paclitaxel. Apart from the cell shown in Fig. 5 B, our studies were done on cells not treated with drugs. In preliminary studies, we have noted greater recovery of EGFP–topoII/H9251 after photobleaching GT2-LPk cells treated with either paclitaxel or nocodazole (unpublished data). Full resolution of the differences between the two studies will require further investigation.

Conclusions
Cell fractionation studies originally suggested that the bulk of topoIIα in mitotic cells was stably associated with the mitotic chromosomes and formed a structural scaffold. Although our evidence of rapid exchange of the bulk of chromosome-associated topoII does not rule out a structural role for the enzyme, any such role must accommodate the fact that most of the protein is highly dynamic, exchanging with the soluble pool in seconds. Consistent with the earlier findings on the single isoform of topoII in Drosophila embryos (Swedlow et al., 1993), we find in mammalian cells a net movement of topoIIα from the chromosomes to the cytoplasm in anaphase. However, two-way exchange between the chromosome arms and cytoplasm persists during anaphase. We report a novel association of topoIIα with NDFs in late mitosis. These organelles may serve to accumulate proteins to newly forming nucleoli in the daughter cells. The rapid dynamics of topoIIα throughout the mitotic chromosomes may be essential in allowing the enzyme to reach and rapidly relieve strain before torsional stresses imparted by chromosome condensation and chromosome movements cause breaks to the DNA.

Materials and methods
Cell culture and immunofluorescence
LLC-Pk were grown in DME supplemented with 10% FBS and antibiotics. To produce a stable cell line expressing EGFP–topoIIα, LLC-Pk cells were transfected using Lipofectamine Plus according to the manufacturer’s instructions. Cells were selected for resistance to 2 μg/ml G418 and cloned by dilution in microwell plates. Cells were processed for immunofluorescence using methods described previously (Kallio et al., 1998).

Immunoprecipitation and decatenation assays
GT2-LPk cells were lysed in high salt buffer (20 mM Tris, pH 8.0, 0.75 M KCl, 0.75% NP40) and immunoprecipitated with anti-EGFP antibody using previously described methods (Kallio et al., 1998). Decatenation assays were performed using a kit supplied by Topogen according to the manufacturer’s directions.

Western blotting
Proteins were separated by SDS polyacrylamide gel electrophoresis using 4–20% gradient acrylamide gels. Proteins were transferred to Immobilon-P...
membrane. Anti-topoIIα was used at 1:1,000. Blots were then incubated with HRP-conjugated goat anti-rabbit and proteins visualized by chemiluminescence and imaged with a digital camera.

**Laser photobleaching**

Cells were observed on a warm stage using a Zeiss Axiosplan II microscope equipped with the ORCA II CCD camera (Hamamatsu) and controlled with MetaMorph software (Universal Imaging Corporation). A nitrogen laser-pumped dye laser (Photonics Instruments) delivered 4 ns laser pulses at 440 nm attenuated with a neutral density slider. Typically, we bleached for 1 s at a repetition rate of 30 Hz. For each time-lapse series in Figs. 3, 4, and 5, the individual frames were imaged and adjusted for contrast using identical conditions.

**Data analysis**

Metamorph software was used for data collection and analysis. Data were analyzed essentially as described (Gorbsky et al., 1990; Howell et al., 2000; Phair and Misteli, 2000). Briefly, an area slightly larger than the target area was used for local cytoplasmic background subtraction. Integrated intensity of the target and background regions and their associated area measurements were logged to Excel spreadsheets. Acquisitions were normalized to adjust for reduction of total cellular fluorescence due to photobleaching and image acquisition (Phair and Misteli, 2000). Normalized relative fluorescence intensity values were exported to GraphPad Prism software and analyzed by nonlinear regression. The rate constant k was determined using the perturbation-relaxation equation and half time of recovery was determined using 1/2 = ln2/k. Percent recovery was calculated according to the method described by Howell et al. (2000) using normalized relative intensity values.

**Online supplemental material**

Videos 1 and 2, corresponding to Figs. 2 A and Fig. 5 B, respectively, are available online as Quicktime movies at http://www.jcb.org/cgi/content/full/jcb.200202053/DC1.

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