Using a Biochemical Approach to Identify the Primary Dimerization Regions in Human DNA Topoisomerase IIα*

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Eukaryotic topoisomerase II is a nuclear enzyme essential for DNA metabolism and chromosome dynamics. The enzyme has a dimeric structure, and subunit dimerization is vital to the cellular functions and activities of the enzyme. Two biochemical approaches based on metal ion affinity chromatography and immunoprecipitation have been carried out to map the dimerization region(s) in human topoisomerase IIα. The results demonstrated that two regions spanning amino acids 1053–1069 and 1124–1143 are both essential for dimerization. The regions correspond to the interaction domains revealed in yeast topoisomerase II after crystallization of a central fragment of this enzyme, indicating that the overall C-terminal dimerization structure of eukaryotic topoisomerase II is conserved from yeast to human.

Furthermore, linker insertion analysis has demonstrated that the two dimerization regions are located in a highly flexible part of the enzyme. Topoisomerase IIα mutant enzymes unable to dimerize via the C-terminal primary dimerization regions due to lack of one of the defined dimerization regions can still be forced to dimerize if DNA and an ATP analog are added to the reaction mixture. The result indicates that secondary interactions occur by ATP analog-mediated clamp closing when the subunits are brought together on DNA.

Eukaryotic topoisomerase II is a nuclear enzyme involved in regulating the topological conformation of DNA (1, 2). It fulfills essential functions during DNA replication (3, 4) and chromosome segregation in both mitosis (5, 6) and meiosis (7), and it is thought to play a key role in certain types of DNA recombination events (8–12). The enzyme has furthermore been suggested to constitute a component of the nuclear scaffold (13, 14), where it is involved in chromosome condensation (15) and decondensation (16).

Mammalian cells contain two structurally similar but biochemically distinct topoisomerase II isoforms, topoisomerase IIα and IIβ, existing in either a homo- or heterodimeric configuration (17–19). The dimeric nature of topoisomerase II is essential to enzyme function. Thus, during the catalytic cycle of the enzyme, it introduces a transient double-strand break into the DNA backbone, where the subunits become covalently linked to the 5’-ends of the respective DNA strands through O4-phosphotyrosine bonds. A second DNA duplex is then transported through the cleaved DNA before it is finally religated (1, 20, 21).

The functional implications of a dimeric nature of eukaryotic topoisomerase II have been supported by several observations. Hydrodynamic studies indicate that the enzyme exists in solution as a dimer (18, 19, 22), and this has further been strengthened by studies of topoisomerase II heterodimers, which have been found to be highly stable in vitro without the ability to dissociate and produce enzymes with a homodimeric conformation (19, 23). Furthermore, structural data obtained either from crystallization of a 92-kDa fragment of yeast topoisomerase II or from electron microscopy studies of human topoisomerase IIα have demonstrated a dimeric nature of these enzymes (24, 25). Despite the strong evidence in favor of a dimeric structure of eukaryotic topoisomerase II, biochemical data on a mapping of the exact regions involved in subunit interaction are still lacking.

Early analysis of the amino acid sequence of human topoisomerase IIα revealed the presence of a leucine zipper (26), a motif that has been shown to selectively direct homo- and heterodimerization of proteins by forming a coiled coil structure, and it was suggested that topoisomerase II dimerization could be mediated through this motif. The importance of the leucine zipper in topoisomerase II dimerization has, however, been questioned by the absence of a similar motif in human topoisomerase IIβ (27), as well as by results obtained by Kroll et al. (28), showing that disruption of the leucine zipper did not have any effect on topoisomerase II dimerization. The same group detected by use of topoisomerase IIα fragments in far Western blotting a region covering amino acids 951–1042 to constitute a minimal subunit association region, whereas maximal homodimerization required sequences C-terminal to position 1042 (29). Similar, another study, using a small human topoisomerase IIα peptide with the ability to adopt a coiled coil structure, has demonstrated that this peptide, covering amino acids 1013–1056, forms a stable homodimer in solution (30).

The most compelling data on eukaryotic topoisomerase II dimerization so far have derived from resolution of the crystal structure of a 92-kDa fragment of yeast topoisomerase II (31). This structure shows a primary interaction domain in the C-terminal part of the fragment involving two minor regions covering amino acids 1031–1046 and 1114–1130 in the yeast topoisomerase II enzyme.

In the present study, we have mapped the regions involved in primary dimerization of human topoisomerase IIα using two biochemical approaches based on metal ion affinity chromatography and immunoprecipitation. The defined sequences are located in the C-terminal region of the enzyme and correspond to the interaction domains discovered in yeast topoisomerase II from crystallization of the central fragment of the yeast en-
zyme. A further investigation of human topoisomerase IIα mutant enzymes lacking the primary C-terminal dimerization region has shown that such enzymes are still capable of dimerization via secondary dimerization regions if DNA and an ATP analog are present.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—The yeast strains BJ4642 (MATa ura3 trp1 leu2 2pep4: HIS3 prb1 can1) and BJ201 (MATa ura3 trp1 leu2 2pep4: HIS3 prb1 can1 top2:TRP1) were used for expression of topoisomerase II constructs. Plasmids pBY1050 and pBY1060 contain the yeast trisoe phosphate isomerase promoter inserted into the LEU2/ARS-CEN plasmid pRS315 and the URA3/ARS-CEN plasmid pRS316 (32), respectively, were used as backbone for introduction of hC1121, hC1123, h1121-12, and wild-type TOP2 cDNAs into *Saccharomyces cerevisiae*. Plasmid pYX212 (purchased from R&D Systems) containing the yeast trisoe phosphate isomerase promoter and the 2-μm origin was used as backbone for introduction of the cDNA constructs hC1053-1069 and hA1124-1143 into *S. cerevisiae*. Construction of the plasmids pHT300, hC1121, hC1123, and h1121-12 is described in Ref. 33. Construction of hA1053-1069 was carried out using the 5′ polymerase chain reaction primer 5′-TGGCTCTAGGAGTGTGTTGCACT- TGGCTAATCTGAATCAGGGCTCGTTAAGAATTAATTAAAGTG- TGTCCTTTTCTTATTTTTG-3′ having the topoisomerase II AvrII site at the 5′ end and the 3′ primer 5′-GGACTTTTTCCCCAATG-3′ having the TopoIIAvrII site at the 3′ end. The resulting AvrII-PstI fragment was subsequently used to substitute the normal AvrII-PstI fragment present in topoisomerase II. Construction of hA1124–1143 was carried out substituting an Xhol-PstI fragment of hA121–2 (35) by a duplex made from the two oligonucleotides 5′-TGCGAGCCACCTGCA-3′ and 5′-GGTGCATGC-3′ and having 5′ Xhol and 3′ PstI overhangs. All constructs have been sequenced to verify the introduced mutations.

**Yeast Transformation and Complementation**—Yeast cells were transformed by using a modified version of the LiAc method of Ito et al. (34). To test the ability of the mutated topoisomerase II constructs to complement the lack of endogenous topoisomerase II in BJ301 the LEU2-based constructs were transformed into BJ301, and cells were transferred to medium plates containing 5′ fluoroorotic acid (1 mg/ml) to select against the UR3 plasmid carrying the *Schizosaccharomyces pombe* TOP2 gene (33).

**Metal Ion Affinity Chromatography**—Single- and double-transformed yeast cells were grown in 50 ml of selection medium to late log phase and were transferred to 1 liter of buffer (4). The cells were harvested approximately 12 h before harvesting by centrifugation. Yeast cells were extracted with 2 volumes of extraction buffer (50 mM Tris-HCl, pH 7.8, 1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and 1 volume of acid-washed glass beads (425–600 μm, Sigma) by vortexing at 4 °C for 30 min. Glass beads and cell debris were removed by centrifugation at 3000 rpm for 10 min, and the supernatant was further centrifuged for 20 min at 12,000 rpm. After filtration (0.65 μm pore size filters), the crude extract was loaded on a 2-ml Ni2+–nitrilotriacetic acid agarose column (matrix purchased from Qiagen) equilibrated with equilibration buffer (20 mM imidazole, pH 8, 1 mM NaCl, 10 mM KPi, pH 8, 10% glycerol). The column was subsequently washed with equilibration buffer and protein was eluted with elution buffer (1 M imidazole, pH 8, 1 M NaCl, 10 mM KPi, pH 8, 10% glycerol). The resulting AvrII-PstI fragment present in topoisomerase II. Construction of hA1124–1143 was carried out substituting an Xhol-PstI fragment of hA121–2 (35) by a duplex made from the two oligonucleotides 5′-TGCGAGCCACCTGCA-3′ and 5′-GGTGCATGC-3′ and having 5′ Xhol and 3′ PstI overhangs. All constructs have been sequenced to verify the introduced mutations.

**Antibodies and Immunostaining**—The C-terminally tagged human topoisomerase IIα mutants were detected by the mouse monoclonal antibody MYC1–9E10.2 (diluted 1:500), purchased from Genosys. Untagged human topoisomerase IIα versions were recognized by the anti-topoisomerase IIα antibody CRB (Genosys) raised against the C-terminal PstI-PstI fragment of the wild-type enzyme, which contain topoisomerase IIα enzyme (diluted 1:5000). For immunostaining, samples collected from the Ni2+ column were subjected to SDS-PAGE1 on 8.5% polyacrylamide gels. Proteins were transferred to nitrocellulose filters (Schleicher & Schuell, 0.45 μm) and immunostaining was carried out according to the ECL protocol (Amersham Pharmacia Biotech). Horseradish peroxidase-conjugated antibodies were used as secondary antibodies (Jackson Immunochemicals). For studies of secondary dimerization, immunostaining of immunoprecipitated material was performed using the penta-His antibody (Qiagen). Immunoprecipitation—Metal ion affinity chromatography was used to purify topoisomerase IIα mutant enzymes for studies of secondary dimerization was performed using protein A-Sepharose. 250 mg of protein A-Sepharose was extensively washed in distilled water followed by three washes in immunoprecipitation buffer (50 mM Tris-HCl, pH 8, 0.35% Triton X-100, 1 mM EDTA, 100 mM NaCl). The Sepharose was finally dissolved in 5 ml of immunoprecipitation buffer. For each immunoprecipitation reaction, 100 μl of Sepharose was washed in immunoprecipitation buffer supplemented with 2 μg of anti-topoisomerase IIα antibody and incubation overnight at 4 °C with gentle shaking. Coated Sepharose was next washed in immunoprecipitation buffer and used for immunoprecipitation by adding the hA1053–1069 enzyme. Following incubation for 1–2 h at 4 °C with gently shaking, the hC1121 mutant enzyme was added either alone or together with DNA (28-mer duplex containing a strong topoisomerase II recognition sequence (8)) and/or an ATP analog (AMP-PNP, Roche Molecular Biochemicals; final concentration, 500 μM), and incubation was continued for 1–2 h before precipitation by centrifugation at 5000 rpm. The NaCl concentration was throughout the immunoprecipitation reaction kept at 100 mM. To avoid unspecific interaction, the precipitated material was washed four times in immunoprecipitation buffer supplemented with 1 mM NaCl and 0.1% SDS and one time in 10 mM Tris-HCl, pH 7.5. The pellet was further prepared for SDS-PAGE as described above.

**Topoisomerase II-mediated DNA Decatenation**—The activity of topoisomerase IIα mutant enzymes was tested by in vitro decatenation of kinetoplast DNA (kDNA) using yeast extract overexpressing the topoisomerase II enzyme of interest. Extract was incubated with 300 ng of kDNA in 1 mM ATP, 5 mM MgCl2, 1 mM dithiothreitol, 0.5 mM EDTA, 50 mM histidine plus ATP-γ-S (0.8), 120 mM potassium glutamate in a total volume of 20 μl. The samples were incubated for 30 min at 37 °C and subsequently subjected to electrophoresis on a 0.7% agarose gel. The contribution of the endogenous yeast topoisomerase II enzyme to decatenation is negligible, as no decatenation activity was seen in yeast extracts when overexpression of the human topoisomerase IIα enzymes was omitted.

**RESULTS AND DISCUSSION**

**Metal Ion Affinity Chromatography as a Biochemical Method to Identify Interactions between Human DNA Topoisomerase IIα Subunits**—In eukaryotic topoisomerase II, subunit dimerization is a key process that is essential for the function of this highly complex enzyme. To map the regions that participate in dimerization in human topoisomerase IIα, we have taken a biochemical approach employing a previously developed protein-protein interaction assay, which takes advantage of the affinity of metal ions for histidine-containing proteins (19). The assay involves coexpression in yeast of two versions of topoisomerase IIα, one of which is His-tagged, followed by analysis of the extract by metal ion affinity chromatography. The strategy behind the assay is that an untagged topoisomerase II enzyme will be retained on a Ni2+: column only if it has interacted with a His-tagged version. A positive interaction can afterward be visualized if the Ni2+: column fractions are examined by immunostaining.

To first verify the specificity of the assay, a human topoisomerase IIα mutant enzyme truncated at residue 1233 (hΔC1233) and fused at its C-terminal end to a bicomposite tag consisting of a hexahistidine tail, and a c-Myc epitope was

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1. The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
expressed in the yeast strain BJ201. Crude extract was subjected to metal ion affinity chromatography under highly stringent conditions, and samples of the eluted material were analyzed by SDS-PAGE. Immunostaining with anti-c-Myc antibody demonstrated the presence of the histidine-tagged hαC1233 topoisomerase II mutant (top panel) or the untagged wild-type human topoisomerase IIα (bottom panel) were subjected to metal ion affinity chromatography. Collected fractions were analyzed by 8.5% SDS-PAGE followed by immunoblotting with the anti-c-Myc antibody (top panel) or the anti-topoisomerase IIα antibody recognizing a C-terminal epitope in human topoisomerase IIα (bottom panel). Fractions are indicated above the immunostainings. Undiluted and 2- and 5-fold diluted extracts are shown. The imidazole gradient used in the elution step ranges from 20 to 1000 mM as indicated. The majority of histidine-tagged human topoisomerase II was eluted at approximately 120 mM imidazole. α indicates the position of the wild-type human topoisomerase IIα enzyme.

**FIG. 1. Metal ion affinity chromatography specifically traps histidine-containing versions of human DNA topoisomerase IIα.** Immunostainings of column fractions from experiments in which extracts from yeast cells expressing either the His-c-Myc-tagged hαC1233 topoisomerase II mutant (top panel) or the untagged wild-type human topoisomerase IIα (bottom panel) were subjected to metal ion affinity chromatography. Completed fractions were analyzed by 8.5% SDS-PAGE followed by immunoblotting with the anti-c-Myc antibody (top panel) or the anti-topoisomerase IIα antibody (bottom panel). Fractions are indicated above the immunostainings. Undiluted and 2- and 5-fold diluted extracts are shown. The imidazole gradient used in the elution step ranges from 20 to 1000 mM as indicated. The majority of histidine-tagged human topoisomerase IIα was eluted at approximately 120 mM imidazole. α indicates the position of the wild-type human topoisomerase IIα enzyme.

**Importance of the C-terminal Region of Human Topoisomerase IIα in Subunit Dimerization—**Several biochemical studies have implied an important role for the C-terminal region of eukaryotic topoisomerase IIα in mediating subunit interaction (30, 33, 35, 36), although direct biochemical evidence is still lacking. As an initial step to map the dimerization region of human topoisomerase IIα, extracts from yeast cells coexpressing untagged wild-type human topoisomerase IIα and a His-c-Myc-tagged C-terminal truncation mutant of the enzyme deleted at either amino acid 1233 or 1121 were analyzed in the described dimerization assay (Fig. 2 and Table I). When the wild-type enzyme was expressed together with hαC1233, the anti-c-Myc antibody identified hαC1233 as a constituent of the column eluate, as expected. The wild-type form of human topoisomerase IIα was retained on the column as well and eluted in the same fractions as the truncated form, as demonstrated by staining with the anti-topoisomerase IIα antibody (Fig. 2, top panel). It has previously been shown that under the conditions employed, unspecific interaction, as well as multimerization, does not occur at least to a detectable level between topoisomerase IIα dimers (19). The coexistence of the truncated and wild-type forms in the elution profile therefore can only be a result of an interaction between the two different subunits, demonstrating that hαC1233 still carries the region essential for dimerization. When extract from yeast cells coexpressing wild-type human topoisomerase IIα and the C-terminal truncation mutant deleted at residue 1121 (hΔC1121) was analyzed in the dimerization assay, only the tagged hΔC1121 mutant was eluted (Fig. 2, bottom panel). The hΔC1121 enzyme is therefore unable to participate in a heterodimeric configuration, suggesting that the region spanning residues 1121–1233 in human topoisomerase IIα has an important role in directing subunit interaction. However, the obtained result does not rule out the possibility that hαC1121 is able to form homodimers. Furthermore, the possibility cannot be excluded that the observed lack of heterodimerization is caused by an altered or destroyed folding of the hαC1121 enzyme.

**Two Minor Regions Located in the C-terminal Part of Topoisomerase IIα Are Essential for Subunit Interaction—**The interpretation of the results obtained with the C-terminal truncated topoisomerase IIα enzymes is that a region residing between amino acids 1121 and 1233 is required for subunit interaction in human topoisomerase IIα. A further dissection of the dimerization region(s) was performed based on information derived from the crystallization of a 92-kDa central fragment of yeast topoisomerase IIα (31). As evident from the crystal structure a major dimerization interface is present in the C-terminal region of the yeast enzyme involving residues 1031–1046 and 1114–1130. Because dimerization is a highly conserved feature of eukaryotic topoisomerase IIα, it is likely that subunit contacts will be mediated by corresponding regions in topoisomerase IIα enzymes of different eukaryotic origin. Therefore, a human topoisomerase IIα mutant was first constructed carrying a deletion of amino acids 1124–1143 (hΔG1124–1143) equivalent to the most C-terminal dimerization region observed in the crystal structure of yeast topoisomerase IIα (residues 1114–1130). This mutant was coexpressed in yeast together with the histidine-tagged, truncated form of human topoisomerase IIα, hαC1233, which still contains the potential to dimerize. In the experiment, hαC1233 was preferred to the wild-type enzyme, as this mutant can easily be distinguished from the deletion mutant upon SDS-PAGE due to its smaller size. Analysis of the crude yeast extract in the dimerization assay revealed that no heterodimer formation takes place between hαC1233 and the deletion mutant hΔ1124–1143 (Fig. 3, top panel), because only the hαC1233 enzyme was retained on the column. The result strongly suggests that the deletion mutant no longer contains the region required for subunit interaction. This is further supported by a failure of hΔ1124–1143 to sustain mitotic growth of the top2 deletion strain BJ201, as well as an inability of the mutant enzyme to decatenate kinetoplast DNA, demonstrating that hΔ1124–1143 lacks a feature necessary for enzy-
The observation underscores the result obtained from the experiment employing the hΔC1121 mutant (Fig. 2). In contrast to this severely C-terminal truncated mutant, the deletion mutant only lacks 20 amino acids, and it is highly unlikely that this deletion would obstruct the overall tertiary conformation of the enzyme. Therefore, the dimerization inability of hΔC1121 is most probably due to lack of a region essential for dimerization activity (Table I).

**TABLE I**

**Analysis of C-terminal mutants of human topoisomerase IIα**

Schematic representation of the human topoisomerase IIα constructs employed. The names of the constructs are indicated to the left, where the numbers refer to amino acid residues in topoisomerase II. wt-yTOPII and wt-hTOPII illustrate wild-type yeast and human topoisomerase IIα, respectively. C-terminal truncation mutants of human topoisomerase IIα are denoted by hΔC followed by a number reflecting the truncation position. Mutant h1121–12 contains a 12-amino acid linker, indicated as a solid bar, inserted at amino acid position 1121. Constructs with internal deletions are illustrated as hΔ followed by numbers indicating the deleted amino acids. The enzymes are divided into three major domains, where the grey box represents the N-terminal ATPase domain, the open box depicts the central cleavage/religation domain, and the hatched box illustrates the divergent C-terminal region. The crystallized fragment of yeast topoisomerase II is indicated by the black line below the schematic representation of *S. cerevisiae* topoisomerase II. The two minor dimerization regions in yeast topoisomerase II and human topoisomerase IIα are shown as black vertical lines. Deletion of one or both of these primary dimerization regions in human topoisomerase IIα is indicated by Δ and a replacement of the black line with a grey line. Constructs fused to a bicomposite tag consisting of the c-Myc epitope, and a hexahistidine tail is marked with + c-Myc-His6. The abilities of all constructs to mediate dimerization, to complement mitotic growth of the yeast strain BJ201, and to decatenate kinetoplast DNA are indicated by + and − signs. nd, not determined.
subunit interaction rather than incorrect folding.

To establish whether the region corresponding to amino acids 1031–1046 in the yeast enzyme also influences dimerization in human topoisomerase IIα, a second deletion mutant, deleted from amino acid 1053 to 1069, was constructed and tested in the dimerization assay together with hΔC1233. From the immunostain shown in Fig. 3 (middle panel), it is seen that only the hΔC1233 enzyme was retained on the column, revealing that the hΔ1053–1069 deletion mutant had also lost its dimerization ability. In correlation with these data, deletion of residues 1053–1069 abolished the ability of the deletion mutant to complement the top2 deletion strain BJ201 (33), demonstrating that these mutant enzymes are still able to interact with either of these regions or both (Fig. 3 and Table I) is detrimental for dimerization. Our biochemical data therefore leads support to the structural information derived from the crystal structure. Moreover, the overall organization of subunit contacts seems to be conserved from yeast to human.

To address whether an exact spacing between the two primary dimerization regions in human topoisomerase IIα is a prerequisite for dimer formation, we have investigated the dimerization potential of a mutant containing a linker inserted at position 1121. In this mutant, a 12-amino acid linker including two proline residues further separates the two subdomains involved in dimerization (33). The linker mutant h1121-12 was tested for its ability to interact with hΔC1233 in the dimerization assay (Fig. 3, bottom panel; Table I). Immunostaining with the anti-topoisomerase IIα antibody together with the anti-c-Myc antibody identifies two distinct bands of the appropriate sizes in the elution profile (Fig. 3, bottom panel). Because this result demonstrates that insertion of an extensive linker between the two dimerization regions is tolerated without functional consequences on the dimerization ability of the enzyme, a correct positioning of the two minor dimerization domains is not required for proper subunit interaction. It cannot be ruled out that the introduced peptide in the linker insertion mutant loops out in a folded conformation due to the presence of two proline residues, thereby conserving the spacing between the two dimerization regions. However, insertion of either two or seven amino acids, designed not to permit outlooping, at position 1121 in human topoisomerase IIα, does not affect the ability of the enzyme to complement the top2 deletion strain BJ201 (33), demonstrating that these mutant enzymes are still in a dimeric configuration (data not shown). Thus, dimerization of human topoisomerase IIα is resistant to alterations in the length separating the two dimerization regions, suggesting a more flexible structure of the C-terminal part of the enzyme.

Recently, a fragment spanning residues 1109–1163 in S. cerevisiae topoisomerase II was found to interact with the enzymes Sgs1 and Pat1 known to be involved in DNA metabolism (37, 38). It is a somewhat surprising observation that more or less the same regions in S. cerevisiae topoisomerase II are involved in subunit contact as well as in directing other protein-protein interactions. This lends further support to a more accessible and flexible structure of the C-terminal domain in eukaryotic topoisomerase IIα.

In human cells two topoisomerase IIα isoforms are present, which can exist in either a homo- or heterodimeric configuration. To investigate whether the regions responsible for homodimerization of human topoisomerase IIα are identical to the regions through which this isoform interacts with the β isoform, the untagged human topoisomerase IIα mutant hΔ1053–1069 was coexpressed in yeast together with a histidine-tagged wild-type version of human topoisomerase IIβ. Analysis of the extract in the dimerization assay demonstrates that the tagged topoisomerase IIβ enzyme is unable to retain the mutated human topoisomerase IIα enzyme on the column (data not shown). Thus, a topoisomerase IIα mutant deleted in one of the primary dimerization regions is deficient in both homo- and heterodimerization, suggesting that the α isoform can interact with either α or β through the same region(s).

Numerous studies have addressed the mechanism and nature of subunit interaction in eukaryotic topoisomerase II. As predicted from the human topoisomerase IIα cDNA, it has been postulated that a potential leucine zipper motif spanning residues 994–1021 plays a key role in mediating subunit interaction (26). However, point mutations disrupting the leucine re-
peptidase sequence in the enzyme does not influence dimerization (28), which is in accordance with our demonstration of a more C-terminal localization of the dimerization regions. Also, a leucine zipper motif is lacking in the human topoisomerase IIβ isoform (27), which together with the natural occurrence of α/β heterodimers in human cells further rules out the importance of the leucine zipper in human topoisomerase II dimerization (19).

Recently, an in vitro study employing human topoisomerase IIα fragments defined a minimal region between residues 951 and 1042 to be required for homodimerization, whereas amino acids C-terminal to position 1042 were found to be necessary for maximal subunit interaction (29). In addition, a peptide fragment covering amino acids 1013–1056 in the human α enzyme was found to form stable dimeric coiled-coil structures, indicating that this small fragment possesses the structural requirements needed for dimerization (30). These observations, however, are not completely compatible with the structural data derived from the crystallization of yeast topoisomerase II and with our biochemical dimerization data on human topoisomerase IIα, where the identified interaction regions are found more C-terminally. The discrepancy in these results might be due to use of only small peptide fragments of human topoisomerase IIα in the two former in vitro studies. It is possible that such fragments may not be folded in the same way and thereby not exposed when present in the full-length enzyme. Furthermore, in the study by Kroll (29), the methods employed to detect dimerization between topoisomerase II fragments involved conditions of low stringency such as 50 mM NaCl. In contrast, the in vivo dimerization assay applied in our study is carried out in the presence of 1 M NaCl to avoid unspecific interactions as well as topoisomerase II multimerization. It cannot be excluded that our assay might be too stringent to detect weak interactions between subunits of topoisomerase II, which could be identified in the above described in vitro studies. Given that the regions observed from the in vitro experiments locate in the vicinity of the dimerization domains mapped in our study and to those in the yeast crystal, it is likely that they play an important role in stabilizing subunit interaction in human topoisomerase IIα.

**Immunoprecipitation Verifies the Requirement of Two Minor Regions for Subunit Dimerization in Human Topoisomerase IIα—**To confirm the above data and to provide an alternative line of evidence for the localization of the two dimerization domains in human topoisomerase IIα, immunoprecipitation experiments were carried out on topoisomerase IIα from crude yeast extract. For this purpose, extract was prepared from cells expressing the combination of two topoisomerase II enzymes previously tested in the dimerization assay. One of the components is either one of the His-c-Myc-tagged C-terminally truncated enzymes, hΔC1233 or hΔC1121, and the other component is the wild-type enzyme, one of the deletion mutants, hΔ1053–1069 and hΔ1124–1143, or the linker insertion mutant h1121-12. In all cases, the extract was incubated with Dynabeads previously coated with the anti-topoisomerase IIα antibody recognizing the C-terminal residues in human topoisomerase IIα. If both topoisomerase II enzymes present in the extract contain the regions involved in subunit interaction, the enzymes recognized by the antibody coupled to the beads will allow co-immunoprecipitation of the truncated topoisomerase II enzyme. The presence of the truncation mutant in the immunoprecipitated material can subsequently be visualized by immunostaining using the c-Myc antibody. As shown in Fig. 4, the truncated hΔC1233 mutant enzyme co-precipitates with wild-type human topoisomerase IIα (lane 1). When further amino acids are deleted from the C-terminal region to position 1121, the truncated form is no longer precipitated by the wild-type enzyme, indicating a loss of dimerization (lane 2). However, the linker insertion mutant h1121-12 is able to precipitate the hΔC1233 mutant (lane 3), verifying that dimerization is not abolished by insertion of 12 amino acids between the two proposed minor dimerization regions. Finally, neither the hΔ1053–1069 nor the hΔ1124–1143 deletion mutant contains the capacity to bring down the truncated hΔC1233 mutant (lanes 4 and 5), so these deletion mutants are deficient in mediating subunit interaction. The immunoprecipitation experiments were carried out in the presence of 1 M NaCl, providing high stringency and thereby eliminating unspecific interaction or multimerization between the different topoisomerase II enzymes. Moreover, the anti-topoisomerase IIα antibody used for immunoprecipitation is highly specific (19), and immunoprecipitation performed with rabbit preimmune serum provides an additional control (lane 6). Thus, the immunoprecipitation data are in correlation with the results obtained from the dimerization assay.

**Secondary Dimerization in Human Topoisomerase IIα Mutant Enzymes Lacking the Primary Dimerization Region—**Biochemical studies by Wang and co-workers (39–41) have demonstrated that topoisomerase IIα operates as an ATP modulated protein clamp. Based on this work as well as information obtained from studies of the homologous gyrase enzyme, a model has been presented in which an ATP-dependent dimerization region exists in the far N-terminal part of eukaryotic topoisomerase II besides the primary dimerization regions in the C-terminal end. Permanent clamp closure was only detected in the presence of an ATP analog, indicating that ATP binding results in clamp closing, whereas ATP hydrolysis causes enzyme turnover and reopening of the clamp. Another N-terminal interaction face has been suggested from elucidation of the crystal structure of the yeast enzyme, where also this interaction is believed to be ATP-dependent, as well as DNA-dependent (31). Electron microscopy studies have demonstrated that a similar overall conformation, including several dimerization faces, is present in the human topoisomerase IIα enzyme (25).

To test whether mutant topoisomerase IIα enzymes lacking the primary C-terminal dimerization regions are able to dimerize in the presence of an ATP analog due to the existence of a secondary dimerization region (4), an immunoprecipitation experiment was performed using hΔ1053–1069 and hΔC1121, each lacking one of the C-terminal dimerization regions (Fig. 5). In the immunoprecipitation reactions the hΔ1053–1069 enzyme was incubated with protein A-Sepharose coated with the anti-topoisomerase IIα antibody recognizing the outermost C-
terminal region of topoisomerase IIα before hΔC1121 was added. Immunoprecipitation was performed in the absence or in the presence of an ATP analog and/or DNA to study the effect of these molecules on the ability of hΔC1121 to dimerize with hΔ1053–1069 through secondary dimerization regions. As seen from the immunostaining in Fig. 5, secondary dimerization between the two mutant enzymes only takes place if both DNA and an ATP analog is present (lane 1). Precipitation in the presence of ATP analog (lane 2) or DNA (lane 3) alone or in the absence of these cofactors (lane 4) can only be revealed after strong overexposure of the film presented in Fig. 5 and probably relates to unspecific protein-protein interactions. The fact that neither DNA nor the ATP analog by itself is sufficient or if DNA is present, secondary dimerization is too weak to withstand this high salt concentration in the absence of an ATP analog. Thus, our results are consistent with a DNA requirement for secondary dimerization of human topoisomerase IIα enzymes lacking the primary C-terminal dimerization region, in which it is so far unknown whether DNA binding per se is sufficient or if DNA cleavage is required. Alternatively, our results can be explained by DNA-independent dimerization followed by subunit exchange. In this case, the two C-terminal topoisomerase IIα mutant enzymes exist as homodimers already at the time of purification, and heterodimerization is only occurring as a result of subunit exchange. If this holds true, our results indicate that a high level of DNA-dependent subunit exchange occurs when hΔC1121 is added to hΔ1153–1169 coupled to protein A-Sepharose. However, we have earlier demonstrated that subunit exchange does not occur to a detectable level with human topoisomerase IIα (19), which makes this explanation less favorable.

Recently, Maxwell and co-workers (42) presented results obtained with an N-terminal human topoisomerase IIα fragment covering amino acids 1–435. By cross-linking experiments, the fragment was shown to be in a dimeric configuration in the absence of DNA, no matter whether an ATP analog or ATP was present or not. The result suggests that secondary dimerization regions present in the N-terminal fragment allow stable interaction under the low salt conditions used in the experiment. The discrepancy between the results obtained by Maxwell and co-workers (42) and our results, in which DNA is fully required to obtain secondary dimerization, might be caused by the use of very different enzyme fragments in the two experiments. Thus, with the almost full-length enzymes used in our experiments, other regions in the enzymes that are absent in the N-terminal peptide might influence the secondary dimerization of the N-terminal regions. Furthermore, our precipitated material was washed in 1 M NaCl before analysis, which will disrupt weak protein interactions, but as discussed above, if the mutant enzymes dimerize in the absence of DNA, we would have expected to see the same amount of precipitation of hΔC1121 whenever an ATP analog was added, no matter whether DNA is present or not.

Taken together, our study has demonstrated the presence of two small regions in the C-terminal domain of human topoisomerase IIα spanning residues 1053–1069 and 1124–1143, each of which is essential for homodimerization of the enzyme. The overall C-terminal dimerization structure is conserved from yeast to human, although the regions involved are located in a highly flexible domain of the enzyme. In our study, deleting part of the C-terminal interaction region abolished the dimerization ability of the enzyme, indicating that if secondary interfaces exist in the protein in the absence of ATP analogs, they are not sufficient to keep the enzyme in a dimeric form under the conditions employed here or, alternatively, that the N-terminal interactions are dependent on a functional C-terminal interaction. However, enzymes lacking the C-terminal dimerization regions can still be brought together if DNA is present. Such dimers are highly unstable in the absence of ATP analogs that ensure clamp closing of the N-terminal domain of the enzyme. Utilization of the biochemical dimerization assay described in this study may allow a dissection of the potential ATP-dependent secondary dimerization regions in human topoisomerase IIα. A complete knowledge on the dimerization faces will aid the further elucidation of the structural organization of topoisomerase IIα and thereby provide valuable information on the catalytic mechanism of this complex enzyme.

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