Identification of a Nucleolin Binding Site in Human Topoisomerase I*

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DNA topoisomerase I (topo I) is involved in the regulation of DNA supercoiling, gene transcription, and rDNA recombination. However, little is known about interactions between topo I and other nuclear proteins. We used affinity chromatography with a topo I fusion protein to screen U-937 leukemic cell extracts and have identified nucleolin as a topo I-binding protein. Coimmunoprecipitation and other studies demonstrate that the interaction between topo I and nucleolin is direct. Furthermore, deletion analyses have identified the 166-210-amino acid region of topo I as sufficient for the interaction with nucleolin. Since nucleolin has been implicated in nuclear transport and in a variety of transcriptional processes, the interaction between topo I and nucleolin may relate to the cellular localization of topo I or to the known role of this topoisomerase in transcription.

Eukaryotic type I topoisomerases are capable of relaxing both negatively and positively supercoiled DNA (1–3). This function is believed to be important in cellular processes requiring access to DNA, such as transcription and replication, and inhibitors of topo I block these events (4–8). However, yeast mutants lacking the enzyme exhibit nearly normal growth, and it has been proposed that other cellular topoisomerases may compensate for the lack of topo I (9). The finding that yeast topo I mutants exhibit an increase in rDNA recombination has supported an irreplaceable role for topo I in maintaining the integrity of the rDNA locus (10). Furthermore, several studies have indicated that topo I is located predominately in the nucleolus (11–14), although the mechanisms related to nucleolar localization of topo I and its unique effects on rDNA are unclear.

In the present studies, we used affinity chromatography with an immobilized fusion protein to identify proteins interacting with topo I. We report that nucleolin, a major nucleolar protein, exhibits binding to topo I in both affinity chromatography and coimmunoprecipitation experiments. A region in the amino terminus of topo I has been identified that is sufficient for the interaction with nucleolin.

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‡Preparation of Immobilized Fusion Proteins and Affinity Chromatography—Escherichia coli strain BLR (Novagene) was utilized for production of topo I fusion proteins. Expression and purification of recombinant GST-fusion protein was as described (15). The bifunctional imidoester dimethylpimelimidate dihydrochloride (Pierce) was used to link the purified fusion proteins or GST alone to glutathione-Sepharose 4B beads (Pharmacia Biotech Inc.). Protein-loaded beads were first washed sequentially with 0.1 M Tris (pH 8.0), 0.1 M K2HPO4 (pH 9.0), and 0.15 M NaCl (pH 9.0) borate buffers. The beads were then incubated for 1 h in 0.15 M borate buffer (pH 9.0) containing 0.01 M dimethylpimelimidate dihydrochloride. After two washes with 0.1 M borate (pH 8.0), the beads were incubated for 45 min in 0.1 M borate containing 40 mM ethanalamide. The cross-linked beads were then washed sequentially with PBS, 0.2 M glycin-HCl, pH 2.5, 1.0 M K2HPO4. These washes were repeated once, followed by two washes with PBS, and the beads were stored at 4°C.

For isolation of topo-I-binding proteins from crude cell extracts, U-937 leukemic cells in exponential growth were collected by centrifugation and washed with PBS. After resuspension in lysis buffer (PBS containing 1.0 mM diithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.1 mM pepstatin), cells were lysed in a Dounce homogenizer and then subjected to brief sonication. After addition of an equal volume of PBS containing 0.2% Tween, the extracts were centrifuged at 15,000 × g for 10 min. The supernatants were added to pre-equilibrated GST-glutathione-Sepharose beads. The flow through and a 1 bed volume wash with PBS, 0.2% Tween were collected and added to pre-equilibrated GST-fusion protein-Sepharose beads. The resulting slurry was incubated for 1 h with rotation, and the beads were then washed twice with 10 bed volumes of PBS, 0.2% Tween. Washing continued with PBS until the A260 of the eluate was <0.01. Bound proteins were eluted with glycine-HCl, and the eluates were neutralized with K2HPO4. The eluates were concentrated, and the buffer was exchanged with PBS, 20% glycerol with the use of Centricon-30 ultrafiltration devices (Amicon). Aliquots of the concentrated eluates were analyzed by SDS-PAGE, with proteins visualized by Coomassie Blue or silver staining.

Protein Microsequencing—In general, the procedures described by Matsuaida (16) were utilized. Samples containing putative topo I binding proteins were subjected to SDS-PAGE and electrophoretically onto 0.45 μm polyvinylidene difluoride membranes (Millipore). The membranes were stained with Coomassie Blue, and selected prominent bands were cut and sequenced from the amino terminus using an Applied Biosystems 477A automated protein sequencer.

Purification of Nucleolin—Nucleolin were prepared from Novikoff hepatoma ascites cells (17). Low ionic strength extracts of the nucleolin were fractionated on heparin-Sepharose columns as described previously (18). Fractions containing greater than 90% nucleolin were used for the nucleolin-topoisomerase I binding studies.

Protein Binding Assays and Immunoblotting—U-937 cell extracts (described above) or purified nucleolin were added to 25-μl aliquots of fusion protein-loaded Sepharose beads in a binding buffer consisting of PBS with 0.2% Tween 20. After incubation at 4°C for 1 h, the beads were washed three times with binding buffer and then twice with PBS. The beads were then boiled in SDS-PAGE loading dye (see above), and released proteins were subjected to electrophoresis. Immunoblotting was performed as described (15), using a monoclonal anti-nucleolin antibody (19).

Coimmunoprecipitation Assays—Approximately 2 × 107 U-937 cells were sedimented and washed with PBS. The cell pellet was lysed by sonication in lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 7.6, 150
m NaCl, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The lysate was centrifuged at 13,000 × g for 15 min, and the supernatant was collected and assayed for protein concentration. Preimmune rabbit serum or anti-nucleolin antibody were added to the cell extract, and the mixtures were rotated for 1 h at 4°C. The mixtures were then incubated with protein A-Sepharose beads for an additional hour at 4°C. The beads were then washed three times with lysis buffer and twice with PBS. The beads were then boiled in protein loading dye, and the released proteins were separated on a 4–15% gradient polyacrylamide gel. Immunoblotting was carried out with a polyclonal topo I antibody (Topogen, Inc.) as described (15).

Construction of GST-Fusion Proteins Containing Topoisomerase I Fragments—pGEX-TOP1 or derivatives of this plasmid were used as a template to make plasmids encoding fragments of the topo I protein linked to the carboxyl terminus of GST. The cloning strategies used to make these plasmids are outlined in Table I. Topoisomerase I Activity Assays—Topo I enzyme activity was measured by a DNA relaxation assay using supercoiled plasmid DNA as described (15).

RESULTS

Identification of Nucleolin as a Topo I-binding Protein—GST-topo I fusion proteins immobilized on Sepharose beads were used to capture cellular proteins binding to topo I. Extracts of U-937 human leukemic cells were first incubated with beads containing GST alone and then with beads containing GST-topo I fusion protein. After extensive washing, bound proteins were eluted, concentrated, and analyzed by SDS-PAGE. Faint bands at approximately 50 and 80 kDa were observed in experiments with cell extracts and beads containing GST alone (Fig. 1). In contrast, several prominent bands of molecular weight (MW) ~65 kDa were observed in experiments with cell extracts and beads containing GST-topo I fusion proteins (Fig. 2). The 65-kDa bands were found to be stoichiometrically linked to topo I in these experiments.

Analysis of topo I-binding proteins in U-937 cell extracts. Crude cell extracts were subjected to affinity chromatography using GST or GST-topo I-Sepharose beads, as described under "Materials and Methods." Bound proteins were eluted and analyzed by polyacrylamide gel electrophoresis, transfer to polyvinylidene difluoride, and Coomassie Blue staining. A photocopy of a representative stained gel is shown. Lanes are labeled according to the matrix used for chromatography, except for the lane labeled PBS, which represents an experiment with GST-topo I beads where PBS was substituted for cell extract. Arrows represent bands that were removed for amino-terminal sequencing.

Table I

Construction of plasmids expressing human topo I fragments

| Plasmid name | DNA template | Cloning strategy |
|--------------|--------------|-----------------|
| Δ1–138       | pGEX-TOP1    | BamHI/XhoI/fill-in |
| Δ1–210       | pGEX-TOP1    | BamHI/EcoRII/fill-in |
| Δ251–765     | pGEX-TOP1    | CellI |
| 227–256      | pGEX-TOP1    | Polymerase chain reaction |
| 231–765      | pGEX-TOP1    | NdeI/BsmI/fill-in |
| 1–139        | Δ231–765     | BamHI/EcoRII/fill-in |
| 140–230      | Δ231–765     | XhoI/EcoRII/fill-in |
| 166–210      | pGEX-TOP1    | Polymerase chain reaction |
| 211–230      | pGEX-TOP1    | Polymerase chain reaction |

Fig. 1. Analysis of topo I-binding proteins in U-937 cell extracts. Crude cell extracts were subjected to affinity chromatography using GST or GST-topo I-Sepharose beads, as described under "Materials and Methods." Bound proteins were eluted and analyzed by polyacrylamide gel electrophoresis, transfer to polyvinylidene difluoride, and Coomassie Blue staining. A photocopy of a representative stained membrane is shown. Lanes are labeled according to the matrix used for chromatography, except for the lane labeled PBS, which represents an experiment with GST-topo I beads where PBS was substituted for cell extract. Arrows represent bands that were removed for amino-terminal sequencing.

Table II

Alignment of 65- and 50-kDa protein sequences with nucleolin

| 65-kDa band nucleolin 227- | K | A | K | N | V | A | E | D | E |
|---------------------------|---|---|---|---|---|---|---|---|---|
| 50-kDa band nucleolin 294-| K | Q | K | N | V | E | G | T | E |

Fig. 2. Immunoblotting of topo I-binding proteins with nucleolin antibody. Cell extracts were subjected to affinity chromatography as described in the legend to Fig. 1. Bound proteins were analyzed by immunoblotting with a monoclonal nucleolin antibody, using a chemiluminescent technique. Lanes are labeled according to the matrix used for chromatography, with the exception of the third lane, which represents direct immunoblotting of U-937 cell extract.

Fig. 3. Topo I coimmunoprecipitates with nucleolin. U-937 cells were lysed by sonication in a buffer containing 1% Nonidet P-40. Aliquots of the soluble cell extract were either loaded directly onto a polycrylamide gel (first lane) or incubated with either a monoclonal nucleolin antibody or preimmune rabbit serum (PIRS), followed by incubation with protein A-linked Sepharose beads. After extensive washing, bound proteins were released by boiling in loading buffer, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with a polyclonal human topo I antibody.

Fig. 4. Binding of nucleolin (Nucn.) to topo I does not involve stoichiometric quantities of other proteins or DNA. A, 5 μg of purified nucleolin were added to 25 μl of covalently linked GST-topo I beads. After washing, bound proteins were released by boiling and analyzed by electrophoresis and silver staining. For comparison, the first lane contains 1 μg of nucleolin alone. B, 2 μg of purified nucleolin were added to 25-μl aliquots of covalently linked GST-topo I beads in a buffer of PBS and 0.2% Tween. In addition, certain reactions contained either DNase I (1 μg/ml) or ethidium bromide (50 or 100 μg/ml), as indicated. After extensive washing, bound proteins were released from the beads by boiling and analyzed by SDS-PAGE and immunoblotting with nucleolin antibody.
masses between 70 and 30 kDa were seen when cell extracts were incubated with GST-topoI beads (Fig. 1). Two prominent bands of approximately 65 and 50 kDa were selected for sequencing. Nine to ten residues were sequenced from the amino terminus of each band, and a database search using the Blast network service from the National Center for Biotechnology Information.

Fig. 5. Analysis of the binding of purified nucleolin to topo I fragments. A, expression of topoI deletion mutants. Aliquots of the indicated purified GST-topoI proteins were subjected to SDS-polyacrylamide gel electrophoresis and Coomassie staining. B, analysis of nucleolin binding to topoI fragments. 2 μg of purified nucleolin were added to glutathione-Sepharose beads containing the indicated fragment of topoI. After extensive washing, the beads were boiled in loading buffer, and released proteins were analyzed by immunoblotting with nucleolin antibody. C, schematic diagram of the results of the binding experiments. The relative levels of nucleolin binding to the topoI fragments are indicated by + signs.

masses between 70 and 30 kDa were seen when cell extracts were incubated with GST-topoI beads (Fig. 1). Two prominent bands of approximately 65 and 50 kDa were selected for sequencing. Nine to ten residues were sequenced from the amino terminus of each band, and a database search using the Blast network service from the National Center for Biotechnology Information.
Information indicated that these sequences were identical to sequences within the human nucleolin protein (Table II). Moreover, the expected electrophoretic mobility of nucleolin fragments containing these sequences at the amino terminus corresponded to that of the sequenced bands. The detection of nucleolin fragments rather than the intact protein is consistent with the known instability of this protein (19, 20). To confirm the binding of nucleolin to topo I, proteins bound to the affinity column were subjected to Western blotting using a nucleolin antibody (19). While no immunoreactive bands were evident when beads containing GST were used in the binding reactions, two bands of approximately 100 and 95 kDa were seen when GST-topo I beads were used (Fig. 2). These bands comigrated with immunoreactive bands obtained using cell extract alone and have been shown to represent intact and degraded forms of nucleolin (21).

To confirm a potential interaction between nucleolin and topo I, we performed coimmunoprecipitation assays. Attempts to immunoprecipitate topo I with a polyclonal antibody from scleroderma patients (22) were unsuccessful (data not shown). However, immunoprecipitates prepared with a monoclonal anti-nucleolin antibody contained proteins of approximately 75 and 67 kDa, which were reactive with the topo I antibody (Fig. 3). Proteins of identical electrophoretic mobility were identified by this antibody in cell extracts, indicating that they represent topoisomerase I fragments. The detection of degraded forms of topo I is consistent with previous results using U-937 cells (15). In contrast to the results obtained with the anti-nucleolin antibody, topo I was not found in control immunoprecipitates prepared with rabbit pre-immune serum (Fig. 3). Thus, binding of nucleolin to topo I is detectable using both affinity chromatography and coimmunoprecipitation.

Characterization of the Binding of Nucleolin to Topo I—To further investigate the interaction between topo I and nucleolin, experiments were performed with purified nucleolin obtained from Novikoff hepatoma cells (18). When this nucleolin preparation was added to GST-topo I beads, a prominent band 100-kDa protein comigrating with nucleolin was detected by silver staining (Fig. 4A). Less prominent bound proteins of higher and lower mobility that were also detected by the silver stain likely represent either nucleolin degradation products or proteins contaminating the nucleolin preparation (Fig. 4A). However, the lack of stoichiometric quantities of these other proteins suggests that they do not serve as intermediaries in the interaction between nucleolin and topo I.

Since both nucleolin and topo I are capable of binding DNA, experiments were performed to determine whether the interaction between nucleolin and topo I involves intermediary DNA. Inclusion of DNase in the binding buffer did not abolish the binding of nucleolin to topo I (Fig. 4B). Similar experiments were performed with ethidium bromide, which is known to abrogate DNA-dependent protein interactions (23). Inclusion of up to 100 μg/ml ethidium bromide in the binding buffer did not affect the binding of nucleolin to topo I (Fig. 4B). These results suggest that DNA is not required for the interaction between nucleolin and topo I. Similar experiments with RNase indicated that RNA is also not involved in this interaction (data not shown).

Identification of a Nucleolin Binding Site in Topo I—To further characterize the binding of nucleolin to topo I, we constructed plasmids expressing various fragments of topo I linked to GST (Table I). Initial constructs involved deletions of either the amino or carboxyl terminus of topo I. In all cases, Coomassie staining of purified preparations of the expressed proteins indicated the presence of an appropriately sized fragment (Fig. 5A and data not shown). However, in some cases smaller proteins were also present, suggesting that the expressed protein was unstable or more susceptible to proteolysis (e.g. Δ251–765, Fig. 5A).

The purified GST-topo I fragments were covalently linked to beads and assayed for nucleolin binding. The results indicate that the first 210 amino acids of topo I are necessary for nucleolin binding and that the first 250 amino acids are sufficient for this interaction (Fig. 5, B and C). The critical residues involved in nucleolin binding appear to be contained in the region 166–210, since a fusion protein containing this region is capable of binding nucleolin, whereas fragments flanking this region are not (Fig. 5, B and C).

Effect of Nucleolin on Topoisomerase I Catalytic Activity—Having identified nucleolin as a topo I-binding protein, we investigated the effect of this binding on topo I catalytic activity in vitro. Purified nucleolin had little, if any, effect on the activity of the topo I fusion protein (Fig. 6). A similar lack of effect was evident in experiments using a lesser quantity of topo I in which there was an equimolar ratio of enzyme and nucleolin (data not shown). These results indicate that under these conditions, nucleolin binding does not have a major effect on topo I catalytic activity.

**DISCUSSION**

The primary cellular function of eukaryotic topoisomerase I is believed to be regulation of DNA supercoiling. Indeed, type I topoisomerasers have been found in nearly every prokaryotic and eukaryotic cell examined and are also found in mitochondria and viruses (2). We sought to gain further insights into the cellular role and regulation of topo I by identifying proteins that interact with this enzyme. Using affinity chromatography and immunoprecipitation, we have identified the 100-kDa nucleolar protein nucleolin as a topo I-binding protein. Previous studies have identified nucleolin as a nonhistone chromosomal protein that localizes to the nucleolus. It is believed to be involved in the regulation of ribosomal RNA transcription and processing. The identification of nucleolin as a topo I-binding protein suggests a potential role for nucleolin in the regulation of DNA supercoiling, which in turn may affect RNA transcription and processing. Further studies will be required to elucidate the functional significance of this interaction.
immunohistochemical studies have indicated that nucleolin and top I are located in similar regions of the nucleolus (25). Furthermore, several studies have implicated top I in the maintenance of both nucleolar structure and function (6, 25–28). The importance of top I in the nucleolus is highlighted by the finding that yeast top I deletion mutants exhibit an increase in rRNA recombination (10).

Our data suggest that the nucleolin-top I interaction is direct and does not require nucleic acid or other proteins. By depletion analysis we have determined that the nucleolin binding site in top I is contained within the first 210 amino acids and that the 166–210 region is sufficient for this binding. This region consists of mostly charged residues, with a slight predominance of basic amino acids and a predicted pl of 8.7. These characteristics are consistent with the finding that nucleolin binding to top I is impaired at pH 10.0 and in solutions of greater than 0.25 M NaCl (data not shown). Although nucleolin is an acidic protein, charge alone does not appear to be sufficient to determine binding of nucleolin by top I peptides, since other basic fragments, e.g. the 140–167 and 1–139 regions (predicted pl values of 8.3 and 9.2, respectively) do not bind nucleolin. Similar results have been reported in analyses of the binding of basic peptides containing nuclear localization sequences (NLSs) by the nucleolar protein B23, in which charge alone was found to be insufficient to determine binding (29). Further analysis of the 166–210 region of human top I indicates that while it is highly conserved among higher eukaryotes, it is only partially conserved in yeast (Table III). This suggests that the nucleolin-top I interaction may be species specific. Indeed, to our knowledge nucleolin has been identified only in higher eukaryotes.

The finding that nucleolin appears to have little effect on top I catalytic activity is consistent with results indicating that deletion of the first 209 amino acids of top I (and thus the nucleolin binding site) does not impair plasmid relaxation activity (data not shown). Moreover, a 67-kDa carboxyl fragment of human top I (30) and a 141–201 deletion mutant of Saccharomyces cerevisiae top I (31) have both been shown to possess a catalytic activity similar to that of the intact protein. If nucleolin binding does not have a significant effect on top I catalytic activity, then what is the purpose of this interaction? Although further study is required to answer this question, several hypotheses may be generated in view of the known functions of nucleolin. This protein constitutes approximately 5% of the total nucleolar protein and has been implicated in nucleolar organization, including rRNA synthesis and shutting of nucleolar proteins from the cytoplasm to the nucleolus (32–35). In addition, the central region of nucleolin contains four consensus RNA binding motifs, which are likely involved in rRNA processing or ribosome assembly (18, 32). Nucleolin has also been assigned more general nuclear roles, including involvement in nuclear transport (34), chromatin decondensation (33), and in the formation of the nuclear matrix (36). Furthermore, nucleolin has been shown to be a transcriptional repressor for the alpha-1 acid glycoprotein gene (37). Taken together, these findings indicate that nucleolin is involved in several aspects of nuclear structure and transcriptional regulation.

Since top I is likely involved in regulation of supercoiling during transcription, one possible role for nucleolin binding is to target top I to sites of transcription. This hypothesis is consistent with prior work indicating interactions between top I and other transcription proteins, such as RNA polymerase I (38), TATA-binding protein (39), and high mobility group proteins (40). Alternatively, nucleolin binding to top I may be the mechanism by which top I enters the nucleus. Indeed, deletion of the 140–230 region in yeast top I results in loss of the nuclear localization of the protein, indicating that NLSs reside in this region (31). Since the corresponding region in the human enzyme includes the 166–210 segment, it is possible that nucleolin binding is required for the nuclear localization of the human enzyme. In this regard, it is important to note that while the 166–210 region of top I does contain a putative bipartite NLS (24), the 140–167 and 1–139 regions each contain a potential bipartite NLS and do not bind nucleolin. Experiments with epitope-tagged top I fragments should allow determination of whether the 166–210 region of top I is necessary and/or sufficient for the nuclear and nucleolar localization of top I.

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