Communication

The RNA-splicing Factor PSF/p54<sup>nrb</sup> Controls DNA-Topoisomerase I Activity by a Direct Interaction* (Received for publication, June 26, 1998)
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DNA-topoisomerase I has been implied in RNA splicing because it catalyzes RNA strand transfer and activates serine/arginine-rich RNA-splicing factors by phosphorylation. Here, we demonstrate a direct interaction between topoisomerase I and pyrimidine tract binding protein-associated splicing factor (PSF), a cofactor of RNA splicing, which forms heterodimers with its smaller homolog, the nuclear RNA-binding protein of 54 kDa (p54<sup>nrb</sup>). Topoisomerase I, PSF, and p54<sup>nrb</sup> copurified in a 1:1:1 ratio from human A431 cell nuclear extracts. Specific binding of topoisomerase I to PSF (but not p54<sup>nrb</sup>) was demonstrated by coimmunoprecipitation and by far Western blotting, in which renatured blots were probed with biotinylated topoisomerase I. Chemical cross-linking of pure topoisomerase I revealed monomeric, dimeric, and trimeric enzyme forms, whereas in the presence of PSF/p54<sup>nrb</sup> the enzyme was cross-linked into complexes larger than homotrimers. When topoisomerase I was complexed with PSF/p54<sup>nrb</sup> it was 16-fold more active than the pure enzyme, which could be stimulated 5- and 16-fold by the addition of recombinant PSF or native PSF/p54<sup>nrb</sup>, respectively. A physiological role of this stimulatory mechanism seems feasible, because topoisomerase I and PSF showed a patched colocalization in A431 cell nuclei, which varied with cell cycle.

DNA-topoisomerase I alters the pitch of DNA double helices by cutting one DNA strand and allowing passage of the complex. The enzyme releases torsional stress in the vicinity of replication forks (2) and transcription complexes (3). Topoisomerase I may also be involved in DNA recombination (4), DNA repair (5, 6), and chromosome formation (7), and it acts as a transcription factor (8) and a protein kinase (9) in a manner not involving DNA turnover. It is not clear how the enzyme is assigned to these multiple functions. Specific binding to various other nucleoproteins, such as RNA polymerase I (10), casein kinase II (11), nucleolin (12), p53 (13), and SV40 large tumor antigen (14) suggests that topoisomerase I may get recruited by protein-protein interactions to sites where its activity is required. On the assumption that such a regulatory mechanism is generally employed, we have searched for new proteins interacting with topoisomerase I and investigated their effects on catalytic DNA turnover by the enzyme.

EXPERIMENTAL PROCEDURES Recombinant Proteins—Topoisomerase I was expressed in Saccharomyces cerevisiae and purified as described (15). Pyrimidine tract binding protein-associated splicing factor (PSF) was expressed in Escherichia coli and purified as described (16).
Protein Purifications from A431 Cells—Topoisomerase I, PSF, and p54<sup>nrb</sup> (nuclear RNA-binding protein of 54 kDa) (17) were copurified from human A431 epidermoid cells (ATCC no. 1555) as follows. Nuclear extracts (20 mg protein) prepared from A431 cells in log growth as described (7) were digested with 3000 units of DNase I for 20 min at 20 °C and passed through a column (0.5 × 1 cm) of Ni-NTA-agarose (Qiagen, Hilden, Germany). The Ni-NTA-column was washed with 20 and eluted with 200 mM imidazole in buffer A (30 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM PMSF, 10% glycerol). The Ni-NTA eluate was passed through a Source-15Q column, adsorbed to a Source-15S column (both Pharmacia Biotech, Uppsala, Sweden), and finally eluted with 400 mM NaCl in buffer A. Topoisomerase I was separated from the PSF/p54<sup>nrb</sup> heterodimer by adding 1 mM ammonium sulfate to the trimeric coeluate of Ni-NTA-agarose. The precipitate contained mainly PSF/p54<sup>nrb</sup> heterodimer, whereas topoisomerase I remained solubilized. Precipitated PSF/p54<sup>nrb</sup> heterodimer was renatured and purified by gel permeation chromatography in the presence of 1 mM NaCl using a Superdex 200 HR30/10 column (Pharmacia Biotech). Peak fractions were dialyzed against 15 mM HEPES, pH 7.5, 300 mM NaCl, 1 mM PMSF, and 50% glycerol. Native endogeneous topoisomerase I soluble in 1 mM ammonium sulfate was adsorbed to phenyl-Sepharose (Pharmacia Biotech), eluted with 30 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM PMSF, 50% glycerol. Analytical gel permeation chromatography of coeluates of topoisomerase I, PSF, and p54<sup>nrb</sup> was carried out in the presence of 400 mM NaCl with or without 1 mM urea using a Superdex 200 HR30/10 column (Pharmacia Biotech).
Protein Chemistry—For N-terminal microsequencing, protein bands were cut out from SDS gels, subjected to BrCN cleavage, re-electrophoresed, transferred to polyvinylidene difluoride membranes, and sequenced from the blots by standard procedures using the automated sequencing apparatus (Applied Biosystems model 476A). Protein cross-linking reactions at 20 °C included 0.1–0.3 mg/ml protein (in 15 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM PMSF, and 10% Me<sub>2</sub>SO) and 0.5 mM SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, Pierce) and were stopped with 100 mM NH<sub>4</sub>Cl and dithiothreitol after 20 min. Cross-linking products were separated in 4% SDS-polyacrylamide gels and analyzed by immunoblotting of topoisomerase I. Recombinant human topoisomerase I was biotinylated using N-biotinyl-e-aminoacproic acid hydroxysuccinimidyl ester (Boehringer Mannheim) without affecting catalytic activity. Immunooassays—Indirect immunofluorescence microscopy of topoisomerase I in A431 cells using human Sc1-70 autoantibodies has been described (7). PSF was visualized similarly using a rabbit antisemur against a C-terminal peptide of human PSF (16), which was also em-
ployed for immunoblotting of PSF, whereas immunoblotting of topoisomerase I followed established procedures (7, 18) using the mouse monoclonal antibody NK 147 (18). Coimmunoprecipitation of topoisomerase I and PSF from chromatographic fractions (200 µl) was achieved by addition of Scv-70 autoantibodies (IgG fraction) to a final concentration of 1 µg/ml and 0.05% Nonidet P-40 and NaCl to a final concentration of 400 mM. After incubation for 1 h at 20 °C, 10 µl of protein A-Sepharose were added (Pharmacia Biotech, equilibrated with PBS containing 1% casein and 0.05% Nonidet P-40), and incubation continued for 1 h at 20 °C. Finally, protein A-Sepharose was sedimented, washed, and eluted with 2% SDS, and eluates were analyzed by immunoblotting with PSF antibodies. For far Western blotting, the blots were renatured for 12 h at 20 °C with PBS containing 0.1% Tween 20 (PBS-Tween) and 5% fat-free milk. After renaturation the blots were incubated for 1 h at 20 °C with the biotinylated enzyme (10 ng/ml in PBS-Tween containing 2% bovine serum albumin). After washing (PBS-Tween + 0.5 mM NaCl) bound enzyme was detected with alkaline phosphatase-conjugated streptavidin (Avidix™) and a chemiluminescent reagent.

**Catalytic Assays**—Kinetic determinations of specific topoisomerase I activities were performed with 2.5 ng of pure topoisomerase I or equivalent amounts of the enzyme contained in other preparations and 0.3 µg of supercoiled pUC18 DNA. Reactions were carried out in a final volume of 20 µl of assay buffer (10 mM BisTris buffer, pH 7.9, 50 mM KC1, 10 mM MgCl2, 0.5 mM EDTA, 0.3 mM dithiothreitol) at 37 °C and were stopped by addition of 1% SDS at the time points indicated. Samples were digested with proteinase K (0.1 mg/ml, 37 °C, 30 min). Relaxed and supercoiled plasmid forms were separated by submari agarose gel electrophoresis, stained with 0.5 µg/ml ethidium bromide, visualized by UV transillumination, and photographed. Images were scanned, and the relative amounts of relaxed DNA were estimated using the image analysis software NIH Image v.1.61. One unit of topoisomerase I activity was defined as the amount of enzyme required for relaxation of 150 ng of supercoiled pUC18 DNA within 30 min. Concentrations of pure recombinant topoisomerase I and pure recombinant human PSF were determined according to Bradford and served as a standard for determining the respective protein concentrations in crude nuclear extracts, preparations of PSF/p54 heterodimers, and trimeric coeluates by comparative immunoblotting. To test the effect of PSF or PSF/p54nrb on topoisomerase I activity, 2.5 ng of pure enzyme or an equivalent amount of topoisomerase I-PSF/p54nrb coeluate was preincubated (15 min, 37 °C) with 120 ng of pure recombinant human PSF or an equivalent amount of isolated native PSF/p54nrb dimer in a final volume of 20 µl of assay buffer.

**RESULTS**

**Interaction of Topoisomerase I to PSF and p54nrb**—Topoisomerase I can be purified from ammonium sulfate precipitates of human nuclear extracts by a single step of nickel affinity chromatography (9). By the same principle, we copurified here a set of three proteins (54, 100, and 115 kDa) from A431 cell nuclear extracts not subjected to high ammonium sulfate concentrations before chromatography (Fig. 1a, lanes 1–3). Copurification in a constant stoichiometric ratio of 1:1:1 was conserved during several other chromatographic procedures including anion exchange (Fig. 1a, lanes 4 and 5), cation exchange (Fig. 1a, lanes 6 and 7), and gel permeation chromatography (Fig. 1b, top) but was disrupted by 1 M urea (Fig. 1a, lanes 8 and 9; Fig. 1b, bottom) or 1 M ammonium sulfate (Fig. 4a, lanes 2’ and 4’), suggesting complexation of these three proteins rather than similar matrix interactions. The 100-kDa protein was identified as topoisomerase I by immunoblotting (Fig. 2a, lane 5), whereas the two other proteins were identified by N-terminal microsequencing. From the 115-kDa protein three peptide sequences identical to amino acid residues 4–21, 214–219, and 685–693 of human PSF (16) were obtained. The identity of PSF was subsequently confirmed by immunoblotting (Fig. 2a, lane 6).

**Fig. 1.** Copurification of topoisomerase I (TopI), PSF, and p54nrb (Coomassie-stained SDS-polyacrylamide gels of chromatographic fractions), a, nickel affinity, anion, and cation exchange chromatography. Lane 1, crude A431 nuclear extract (NE); lanes 2, 4, 6, and 8, unbound fractions (P); lanes 3, 5, 7, and 9, bound/eluted fractions (E); lanes 2 and 3, Ni-NTA-agarose chromatography of nuclear extracts (Ni); lanes 4 and 5, rechromatography of nickel eluates (same as lane 3) with Source15-Q (Q); lanes 6–9, chromatography of the unbound fraction of Source15-Q (same as lane 4) with Source15-S in the absence (S) or presence of 1 M urea (S+Urea); lane 10, marker proteins. b, gel permeation chromatography (Superdex 200) of eluates from Ni-NTA-agarose without (top) or with (bottom) 1 M urea. SM, starting material. Lanes 1–10, effluent collected in fractions of 1 ml subsequent to the void volume. Markers on the left indicate the positions of PSF, topoisomerase I, p54nrb, and an unknown contaminating protein (Cont) serving as endogenous marker of the separation.

From the 54-kDa protein three peptide sequences identical to amino acid residues 4–14, 90–120, and 443–466 of human nuclear RNA-binding protein p54nrb (17) were obtained. PSF and topoisomerase I could be coimmunoprecipitated with topoisomerase I antibodies (Fig. 2b, lane 3), and a specific binding of pure recombinant human topoisomerase I to PSF but not to p54nrb could be demonstrated by far Western blotting, probing renatured Western blots of crude nuclear extracts (Fig. 2a, lane 3) or coeluates of topoisomerase I, PSF, and p54nrb (Fig. 2b, lane 4) with biotinylated topoisomerase I. From the experiment in Fig. 2a, lanes 3 and 4, it became apparent that topoisomerase I also binds strongly to itself, which suggests multimerization of the enzyme. Treatment of pure recombinant human topoisomerase I (Fig. 2c, lane 5) with SMCC stabilized fractions of the enzyme in dimeric (30%) or trimeric (10%) complexes (Fig. 2c, lane 1), when coeluates containing topoisomerase I, PSF, and p54nrb but no other proteins (Fig. 2c, lane 6) were treated with SMCC, similar fractions of dimeric and trimeric topoisomerase I were obtained, but in addition, the enzyme became cross-linked into complexes much larger than homotrimers (Fig. 2c, lane 2). These data indicate that human topoisomerase I binds PSF, which in turn is associated with p54nrb.

**Colocalization of Topoisomerase I and PSF in A431 Cells**—To determine whether such complexes could also assemble in the cell, we double stained topoisomerase I and PSF in human A431 cells and compared their cellular localization patterns by indirect immunofluorescence microscopy (Fig. 3). During most of mitosis the two proteins were separated because...
PSF diffused into the cytosol in prometaphase (Fig. 3a), remained excluded from the chromatin during metaphase (Fig. 3b) and early anaphase (Fig. 3c), and subsequently reassociated with the DNA in late anaphase (Fig. 3d) and telophase (Fig. 3e). Topoisomerase I, on the other hand, remained entirely chromatin-bound during all these mitotic steps. In most interphase cells both proteins had a diffuse nuclear distribution (in the case of PSF strictly excluding the nucleoli) and showed extended patches of colocalization in the extranucleolar nucleoplasm (Fig. 3f, yellow patches), whereas in a minor fraction (5–10%) of interphase nuclei the two proteins showed grainy and inhomogeneous patterns and did not colocalize but, on the contrary, appeared to be compartmentalized away from each other (Fig. 3g). These observations suggest that interactions between topoisomerase I and PSF can occur in the cell and might be governed by cell cycle-related redistribution and compartmentalization.

Stimulation of Topoisomerase I by PSF—Initially, we observed that topoisomerase I of human A431 cells was 8-fold more active in the context of a crude nuclear extract (Table I, line 8) than in the purified state (Table I, line 4). The pure enzyme of A431 cells had a specific activity of $2.9 \times 10^3$ units/µg similar to pure recombinant human topoisomerase I (Table I, line 1) and Ref. 19, whereas in the copurified complex with PSF/p54<sub>nrb</sub> topoisomerase I was even more active than in the nuclear extract (Table I, line 7). Thus, we assumed that the decrease in enzyme activity upon purification could be because of the removal of PSF/p54<sub>nrb</sub>. To test this assumption, we preincubated pure recombinant (Fig. 4a, lane 1) or endogenous topoisomerase I (Fig. 4a, lane 2) with pure recombinant PSF (Fig. 4a, lane 3) or isolated native PSF/p54<sub>nrb</sub> dimer (Fig. 4a, lane 4) and compared it to the copurified topoisomerase I-PSF/p54<sub>nrb</sub> complex (Fig. 4a, lane 5). Such an experiment is shown in Fig. 4b. Quantitative results are summarized in Table I. The isolated native PSF/p54<sub>nrb</sub> dimer, which did not have DNA relaxation activity of its own (Fig. 4b), stimulated the activity of purified endogenous topoisomerase I at least 16-fold (thus entirely restoring the activity of the trimeric complex), whereas it had a slightly lesser effect on recombinant human topoisomerase I. Recombinant human PSF also stimu-
Insignificantly increased by preincubation with a 50-fold molar excess of PSF/p54nrb. Furthermore, we show that the PSF/p54nrb (rhPSF); lane 4, 1.5 µg of PSF/p54nrb isolated from A431 cells; lane 5, 2.5 µg of topoisomerase I-PSF/p54nrb complex, copurified from A431 cells (compare Fig. 1, lane 4; lane 6, marker proteins. a, representative examples of pUC18 DNA relaxation kinetics. Identification of protein preparations on the right are the same as in a. Each assay contained the equivalent of 2.5 ng of topoisomerase I but was 3 times less effective than the native PSF/p54nrb dimer. The activity of topoisomerase I in the copurified equimolar complex with PSF/p54nrb was insignificantly increased by preincubation with a 50-fold molar excess of recombinant human PSF or isolated native PSF/p54nrb dimer (Table I), suggesting that the stimulatory effect was saturated at equimolar ratios of the three proteins. In summary, these data show that PSF/p54nrb is a strong stimulator of topoisomerase I in human A431 cells.

**DISCUSSION**

Human topoisomerase I is involved in the activation of serine/arginine-rich cofactors of RNA splicing, either by an endogenous protein kinase activity (9) or by physical association with such an enzyme (21). Here, we report on a direct interaction of topoisomerase I and another RNA-splicing factor. We show that human topoisomerase I binds PSF, an essential cofactor of the second step of RNA splicing (16), and is indirectly (via PSF (22, 23)) associated with p54nrb, a PSF homolog of unknown function (17). Furthermore, we show that the PSF/p54nrb dimer has pronounced stimulatory effect on DNA catalysis by topoisomerase I in vitro. Thus, PSF/p54nrb could be a regulator of topoisomerase I, independent of and in addition to its functions in RNA splicing. However, because PSF is a well-known RNA-splicing factor, it is also tempting to speculate that the observed interaction between topoisomerase I and PSF/p54nrb reflects a function of topoisomerase I in RNA splicing.

In contrast to stimulation of topoisomerase I by histone H1 and high mobility group proteins (11), which probably reflect an indirect DNA-mediated effect, stimulation of topoisomerase I by PSF/p54nrb was seen with equimolar complexes of these proteins and could not be significantly increased by further addition of the stimulatory proteins at a 50-fold molar excess. Thus, it seems to be mostly because of a direct protein-protein interaction. As we were not able to separate the native PSF/p54nrb dimer, we could not directly assess the individual contribution of p54nrb to this stimulatory effect. We observed that recombinant PSF alone was not as effective as the native PSF/p54nrb dimer. This could be because of the lack of p54nrb but could equally well be because of inappropriate folding of the recombinant PSF or lack of posttranslational modifications. It should be noted that endogeneous PSF of A431 cells had a slightly different migration in a SDS gel than recombinant PSF produced in E. coli (Fig. 4a, lanes 3 and 4).

Partial colocalization of PSF and topoisomerase I in interphase nuclei of human A431 cells (Fig. 3, e–g) suggests that complexes of these proteins could assemble in the cell in a manner regulated by compartmentalization. We have not yet obtained direct evidence that such interactions actually serve the regulation of topoisomerase I in a living cell. However, it should be noted that the complete dissociation of topoisomerase I and PSF during early mitosis shown in Fig. 3, a–d, coincides with a 4-fold drop in topoisomerase I activity extractable from mitotic A431 cells (7).

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