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NEGATIVE REGULATION OF Scl-70/TOPOISOMERASE I BY ZINC AND AN ENDOGENOUS MACROMOLECULE

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Scl-70/topoisomerase I (topo I), a target of autoantibodies in the human disorder scleroderma, may be linked to collagen overproduction. We report that physiologic concentrations of zinc (≥80 μM) potently inactivate human and rat topo I in the presence of a 125-fold molar excess of $MgCl_2$. We also describe a highly acidic nuclear molecule which competitively inhibits topo I. 89kD complexes of topo I and inhibitor can be separated from active 70kD monomers by glycerol density gradient centrifugation. The complexes have a 20-fold higher Km for DNA than monomers. Dissociation by dilution results in a 50-fold increase in rat liver and a 47-fold increase in human fibroblast specific activity. Zinc inhibition is non-competitive, and independent of the endogenous inhibitor. Our studies suggest that the majority of topo I is normally maintained in an inactive state by physiologic inhibitors, and loss of negative regulation may have pathogenetic consequences.

In the rheumatic disorder scleroderma, the primary mechanism responsible for collagen overproduction, and therefore dermal hardening, is accelerated collagen gene transcription (1-2). The DNA unwinding enzyme topo I is essential for gene transcription (3, 4), and can selectively activate some gene clusters (5). We previously suggested that the dermal collagen genes may be among those selectively activated, because potential binding sites for topo I are 4-fold more abundant in these genes than in control gene clusters (6). Binding sequences occur in three areas of regulatory importance: promoters, exon-intron junctions, and 3'-nontranslated ends. The regulation of topo I therefore has pathogenetic significance in disorders of collagen production. A pathogenetic link in scleroderma is further suggested by the presence of high-titer antibodies to topo I, also known as Scl-70 (7), in 25 - 30% of scleroderma patients (8, 9).

Prior to this report, no negative physiologic regulators of topo I had been described. Phosphorylation via a protein kinase type II activates the enzyme in vitro, and probably in living cells (10, 11). Here we characterize an inhibitory ligand from rat liver and human fibroblasts, and describe its kinetic effects on topo I activity. We also present evidence that dissociated topo I

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monomers, having a basic isoelectric point, represent the active form. A second finding reported here is that zinc is a potent and specific inactivator of topo I. This finding is particularly interesting in view of the known anti-fibrotic effects of zinc. Previously reported effects include stimulation of collagenolytic metaloproteinases (12, 13) and inhibition of prolyl hydroxylases (14, 15).

MATERIALS AND METHODS

Topo I from rat liver nuclei was partially purified by Bio-Rex 70 chromatography as described previously (7), and dialyzed extensively against 30 mM potassium phosphate 0.5 mM dithiothreitol, 5% glycerol to remove ions and small molecules. Further purification to monomeric topo I was accomplished by centrifugation through linear 5-20% glycerol density gradients buffered by 10 mM Tris-HCl pH 8.0, 200 mM NaCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol. Separation of monomeric topo I from inhibited complexes was also accomplished using the same glycerol gradient system. Topo I assay mixtures consisted of 20 μl volumes containing 1 μl (0.239 μg) pBR322 DNA (BRL), 1-3 μl topo I, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 12 mM KCl, and 0.25 mM dithiothreitol. The amount of topo I varied according to the requirements of each experiment (figure legends). Incubations were performed for 30 minutes at 37° C. Supercoiled and relaxed forms of DNA were separated by electrophoresis in 1% agarose gels followed by ethidium bromide staining and quantitation of photographed gels by laser densitometry (16).

Human fibroblasts were grown from skin biopsies and cultured in RPMI (Irvine Scientific) supplemented with 10% fetal calf and human serum (2). Nuclei were obtained by lysis of fibroblasts in 0.5 mM $CaCl_2$, 5 mM PIPES, pH 6.8 for 20 min. at 0° followed by addition of 1.1% NP-40. After lysis of nuclei by vortexing, the topo I was extracted with 1 M KCl, 10 mM Tris-HCl buffer, pH 7.5 followed by centrifugation to remove chromosomal DNA and unextracted proteins.

For dilution and mixing experiments, topo I preparations were extensively dialyzed in 30 mM potassium phosphate buffer pH 7.6, 0.5 mM dithiothreitol, 5% glycerol. Denaturing polyacrylamide gel electrophoresis and silver staining were performed according to protocols from refs. 17 and 18, respectively. Western blots of topo I glycerol density gradient centrifugation fractions were performed as previously described (19) using anti-Scl-70 IgG antibodies. Rocket electrophoresis of gradient fractions was performed as described (20). Polyaspartic, polyglutamic acid and immobilized trypsin and RNase A and tRNA were purchased from Sigma.

RESULTS

The effects of zinc and a dissociable inhibitor on rat and human topo I--By Bio-Rex 70 chromatography, rat liver topo I is isolated as a 10% pure, partially active enzyme complexed with other nuclear molecules (7). Agarose gel data presented in Fig. 1A demonstrate partial conversion of supercoiled pBR322 DNA (lane 1) to the relaxed form (lane 2) by this preparation. Dilution of the enzyme by factors of 1:4, 1:10 and 1:40 (lanes 3-5) results in dramatic increases in enzymic activity, and full relaxation of supercoiled DNA, despite the addition of respectively less enzyme to reaction mixtures. Calculations from densitometry scans reveal a 50-fold increase in specific activity at the 1:40 dilution. Lanes 2'- 5' of panel 1B show that addition of 300 μM $ZnCl_2$, in the presence of 10 mM $MgCl_2$, totally inhibits all dilutions of the enzyme. Zinc alone has no effect on DNA configuration (lane 1). Panel 1C demonstrates that similar to the rat enzyme, normal human fibroblast topo I undergoes dilutional activation. An average 47-fold increase in specific activity is seen at 1:20 dilutions (determined by densitometer). Panel 1D (lanes 2' and 3') demonstrates that 1:10 dilutions of both normal and scleroderma fibroblast topo I are highly active, but upon addition of 300 μM $ZnCl_2$, both preparations are totally inhibited (lanes 3' and 4'). Fig. 1 thus demonstrates that zinc inhibition is not dependent on either the species or clinical status of the donor. It was therefore of interest, because of the physiologic implications of negative regulation, to further characterize the specificity of zinc inhibition, and the kinetic effects of the endogenous inhibitor (below).

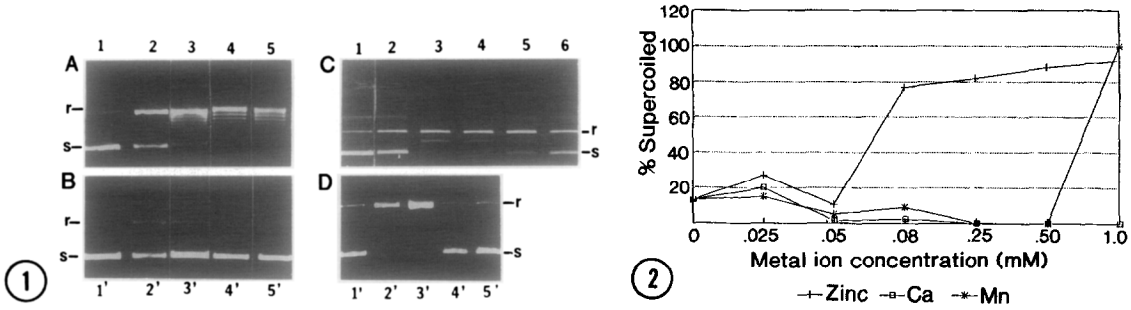

FIG. 1. THE EFFECTS OF DILUTION AND ZINC ON PARTIALLY PURIFIED TOPOISOMERASE I ACTIVITY.

1 μl of rat or 3 μl of human fiborblast topo I and 30 mM potassium phosphate buffer pH 7.6, 0.5 mM dithiothreitol, 55 glycerol was incubated with 0.24 μg pBR322 DNA as detailed in Materials and Methods, followed by electrophoresis through 1% agarose and ethidium bromide staining. The concentration of undiluted rat topo I was approximately 30 μg/ml.

Panel A - Agarose gel assays of rat liver topo I activity. Lane 1 - DNA control without topo I; Lane 2 - DNA incubated with undiluted topo I; Lanes 3, 4, 5 - DNA incubated with 1:4, 1:10, and 1:40 dilutions of topo I. S = supercoiled form of DNA; R = relaxed form.

Panel B - The same assays as panel A, but undiluted topo I and 1:4, 1:10, 1:20 and 1:40 dilutions, respectively.

Panel C - assay of normal human fibroblast topo I, isolated from nuclei as described in Materials and Methods. Lane 1, control pBR322 DNA. Lanes 2-6, undiluted topo I and 1:4, 1:10, 1:20, and 1:40 dilutions, respectively.

Panel D - assay of normal and scleroderma fibroblast topo I with added zinc (300 μM). Lane 1', control DNA with zinc. Lanes 2' and 3', normal and scleroderma topo I added, 1:10 dilutions, no zinc. Lanes 4' and 5', normal and scleroderma topo I with added zinc.

FIG. 2. THE EFFECTS OF ZINC, CALCIUM AND MANGANESE ON MAGNESIUM-ACTIVATED TOPO I.

Rat liver topo I (μl of a 1:1 dilution) was incubated in reaction mixtures containing 10 mM $MgCl_2$ plus the indicated concentrations of $ZnCl_2$, $CaCl_2$ or $MnCl_2$, then assayed by agarose gel electrophoresis as in Materials and Methods. The % pBR322 DNA remaining in the supercoiled form after incubation is indicated. In the control reaction (with $MgCl_2$ as the only divalent cation), 17% of the DNA remained supercoiled.

Zinc specificity and kinetic effects--To determine if the above reported inhibitory effects are specific for zinc, we compared increasing concentrations of $ZnCl_2$, $MnCl_2$ and $CaCl_2$ in the presence of 10 mM $MgCl_2$ (Fig. 2). In 10 mM $MgCl_2$, and a 1:1 dilution of topo I, 17% of the DNA added to reaction mixtures remains supercoiled. At low concentrations ($\leq$0.5 mM) $MnCl_2$ and $CaCl_2$ show a 15% stimulation (2% of the DNA remains supercoiled) whereas a sharp rise in inhibition is seen at 80 μM $ZnCl_2$ (in the presence of a 125-fold molar excess of $MgCl_2$). Inhibition by $MnCl_2$ is seen at 1 mM, a 12.5-fold higher concentration than the effective inhibitory concentration of $ZnCl_2$. $CaCl_2$ was moderately stimulatory throughout the concentration range tested.

The inhibitory effects of zinc are not substrate reversible (kinetic plot not shown). Over a range of 0.23-1.16 μg of pBR322 DNA, the activity of topo I in 10 mM $MgCl_2$ without zinc increased by a factor of 6.2 (from 0.1 to 0.62 μg of DNA converted to the relaxed form). In contrast, no activity was detected with increased DNA concentration in the presence of 0.2 mM $ZnCl_2$ (Vmax=0). These data are consistent with non-competitive inhibition.

Kinetics of inhibition of partially purified topo I by the endogenous inhibitor--This study compared the effects of increasing DNA substrate on a 1:2 dilution (less active) and 1:10 dilution (more active) of topo I. Velocity vs. substrate plots, and Lineweaver-Burk plots of these data (not shown) reveal the same Vmax of $8x10^{-9}$ moles of pBR322/30 min, but different Km values of $5x10^{-7}$ M^{-1} and $2.5x10^{-8}$ M^{-1} for 1:2 and 1:10 dilutions, respectively. These findings are consistent with significant competitive inhibition by a reversibly associated inhibitor. The separation of inhibited complexes from active monomers of topo I is described below.

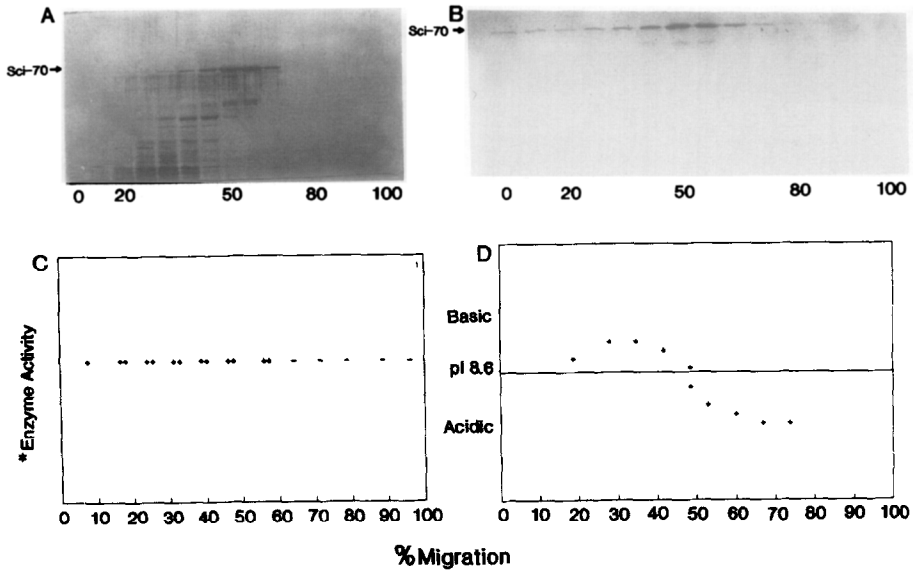

FIG. 3. RESOLUTION OF Scl-70/TOPO I MONOMERS FROM INHIBITED COMPLEXES BY GLYCEROL DENSITY GRADIENT CENTRIFUGATION.

Glycerol density gradient centrifugation (5-20% glycerol) was performed as described in Materials and Methods. % migration (the horizontal axis in all 4 panels, belos) was calculated from the position of each gradient fraction relative to the total number of fractions. Typically, 0.5 ml fractions were collected. SDS polyacrylamide gel electrophoresis was performed as in ref. 17, and silver staining and Western blotting as in refs. 18 and 19.

Panel A - Silver stained gel of each gradient fraction, with 100% migration representing the bottom fraction. The vertical axis represents migration rates with the position of Scl-70 corresponding to a globular protein of 70 kD, as established previously (7).

Panel B - Western blot of each fraction of a 5-20% glycerol gradient. Vertical and horizontal axes are as described in panel A.

Panel C - Topo I activity of each gradient fraction as determine by the DNA relaxation assay described in Materials and Methods. A 1 μl aliquot of a 1:5 dilution of each gradient fraction was added to the assay. The asterisk on the vertical axis denotes the strength of enzymic activity. This was determined by serial dilution of each fraction, with a ++ symbol indicating complete relaxation at a 1:10 dilution of the fraction.

Panel D - Rocket electrophores of gradient fractions performed as described (20). Total human IgG was used as the pI 8.6 standard.

Glycerol density gradient comparisons of Scl-70/topo I protein distribution and antigenic activity. Resolution of enzymically active monomers from inhibited complexes--To further characterize the relation between active enzyme and inhibited complexes, partially purified topo I was subjected to linear glycerol density gradient centrifugation (5-20%). For each gradient fraction we determined the protein distribution (by gel electrophoresis), the antigenic potency (by western blot), the topo I activity (as in Materials and Methods), and the isoelectric characteristics of antigenically active protein (by rocket electrophoresis). These results are summarized in Fig. 3. Panels A and B (a silver stained denaturing gel and western blot, respectively) show that a Mr=70,000 protein, previously determined to consist entirely of Scl-70 (7), migrates anomalously in the 50-60% position of the gradient, corresponding to a size of 89 kD, (based on gradient standardization using globular, monomeric proteins). However, the topo I activity (panel C) occupies a less dense position, centered in the 25-45% region, corresponding to the predicted size of 70 kD monomers (7, 21). These measurements are highly reproducible. The most likely interpretation of the data (discussed in greater detail below) is that an approximately 19 kD ligand is associated with the 70 kD topo I monomer in the heavier region of the gradient. As shown in panel D, there is a

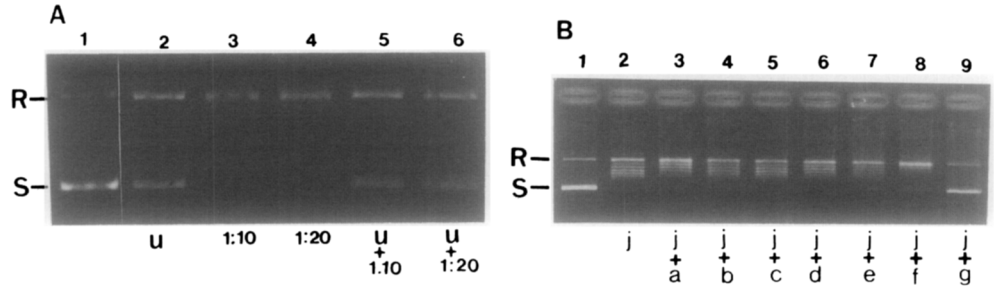

FIG. 4. INHIBITION OF TOPO I ACTIVITY BY MIXING.

Panel A - Assay by agarose gel electrophoresis of rat liver topo I activity from partially purified complexes. Lane 1 - DNA control without topo I: Lane 2 - DNA incubated with 1 μl of undiluted (u) topo I: Lanes 3 and 4 - DNA incubated with 1 μl 1:10 and 1:20 dilutions of topo I; Lanes 5 and 6 - DNA incubated with 1 μl undiluted topo I plus 1 μl 1:10 and 1:20 dilutions of topo I. S = supercoiled DNA; R = Relaxed DNA.

Panel B - Assay by agarose gel electrophoresis of glycerol gradient purified topo I activity. Lane 1 - DNA control without topo I: Lane 2 - DNA incubated with 3 μl of glycerol gradient fraction j. Lanes 3-9 - DNA incubated fraction j plus 3 μl of fractions a-g, where 'a' is the bottom fraction.

dramatic change in the isoelectric point of the antigen between active and inactive forms, indicating that the inhibitory ligand is highly acidic.

Inhibition of topo I by mixing active monomers with inactive complexes--In addition to the dilution effects reported in Fig. 1, the presence of a reversibly associated inhibitor can demonstrated by mixing active and inactive enzyme, as shown in Fig. 4. Experiments using partially purified rat liver complexes (panel A) demonstrate that mixing relatively inactive, undiluted topo I (lane 2) with more active dilutions (1:10 and 1:20, lanes 3 and 4, respectively) results in inhibition of the latter (lanes 5 and 6). Similarly, as shown in panel B, active 70 kD enzyme, obtained by glycerol density centrifugation (fraction j), is inhibited by a fraction (g) obtained for the 89 kD area of the gradient. By mixing fraction j with a series of fractions beginning from the bottom of the gradient (a-g) we confirmed that the inhibitor is in the acidic, 50-60% region of the gradient (89 kD). Incubation of the inhibitor with immobilized trypsin and RNase A failed to destroy the inhibitory activity. Because of its ultimate effect on the DNA substrate in the reaction mixture, it was impossible to pre-treat with DNase I. (Immobilized DNase is not available). Further, addition of polyglutamic acid polyaspartic acid, and tRNA to the active topo I had no inhibitory effect (not shown). The inhibition is therefore not a nonspecific polyanion effect.

DISCUSSION

Human topoisomerase I is a 110 kD molecule, of which a basic 67-70 kD fragment is the catalytically active portion (21). In rat, we previously isolated a 70 kD monomer, analogous to the human catalytic subunit (7). Anomalies in topo I activity (or in the balance between topo I and topo II) are being increasingly associated with human genetic disorders (22, 23). We have postulated that anomalies in the regulation of this enzyme are also linked to the acquired disorder scleroderma (6). In addition to the previously described activation of topo I by phosphorylation (10, 11) regulation may also involve inhibitory ligands present in physiologic levels in nuclei. Here we provide the first experimental evidence for such ligands, including zinc and an endogenous molecule which forms reversible complexes with topo I.

The potent inhibition of topo I by zinc (figs. 1 and 2) is interesting for several reasons. First, it is both an activator of some enzymes (carbonic anhydrase, DNA polymerase) (24-25) and an

inhibitor of others (prolyl hydroxylase, phosphofructokinase) (12, 27). This is that first report that it inhibits topo I. Second, it is a known anti-fibrotic agent, presumably due to its activation of collagenolytic enzymes and inhibition of prolyl hydroxylase (12-15). Its in vivo anti-fibrotic effects include a 24% reduction in skin hydroxyproline in rats (28). Further, in CCl_4-treated rats which develop internal fibrosis of the lung and liver, the resulting $\geq$ 2-fold rise in collagen accumulation is prevented by oral zinc (13, 28). These effects may result not only from post-translational mechanisms, but also from topo I inhibition. Third, our data (fig. 1 and text) indicate that unlike the pharmacologic inhibitory camptothecin, which interferes with dissociation of topo I/DNA complexes (29), zinc acts directly on the enzyme. The distinct and separate mechanisms whereby these two agents inhibit collagen production suggest the potential for synergistic action in a therapeutic setting.

Interestingly, zinc has been tried as a therapeutic agent in other rheumatic disorders, but as an anti-inflammatory, not an anti-fibrotic agent (30, 31). There are no reported trials in scleroderma patients. The feasibility of attaining intranuclear zinc levels which may inhibit topo I is demonstrated by the concentrations (shown in fig. 2) at which we measure total, or near total inactivation of the enzyme (80-200 μM). Zinc is concentrated in organs rather than is extracellular fluid, and nuclei are one of its primary areas of subcellular concentration (32). Skin zinc levels are estimated to be between 100 and 1300 μM (33, 34). Thus the inhibitory effects which we report in Figs. 1 and 2 are below or well within the physiologic range.

Of further regulatory significance is the potential for zinc finger configurations in the NH_2 end of topo I. By computer assisted modeling we have identified several possible structures (not shown) conforming to previously reported consensus configurations for zinc fingers (for reviews see refs. 35, 36). Zinc fingers are associated with hormonally regulated enzymes and receptors. Scleroderma is a female-predominant disorder (a 10:1 female to male ratio), suggesting that hormonal systems play a role in dysregulation of collagen production.

The data presented in figs. 1 and 3 demonstrate that the majority of Scl-70/topo I is in an enzymically inactive form having a greater density (89 kD) than the catalytically active monomer (70 kD). The inactive state is reversible with dilution (fig. 1) and can be reconstituted by mixing (fig. 4). Although not fully characterized, several observations identify the inhibitor as a 19 kD, highly acidic molecule, perhaps a specific nucleic acid: (a) First, neither larger nor smaller than 89 kD inhibited complexes were identified in glycerol gradients. Therefore two other inhibitory configurations can be ruled out. One of these is dimerization of topo I. A second is a proteolytic event which activates the 89 kD molecule to the 70 kD form. This is because rat liver Scl-70/topo I consistently has a Mr=70,000 on denaturing polyacrylamide gels, whether derived from crude enzyme (7) or from 70 kD active or 89 kd inactive enzyme (fig. 3). Finally, the acidity of the inhibitory ligand is such that it changes the net charge of topo I (which has 614 amino acids, including 168 arginine plus lysine residues and a pI>10) to well below that of IgG (fig. 3). Predictions based on the required isoelectric point of the ligand, the known DNA-binding properties of topo I, and the resistance of the inhibitor to RNase A and trypsin suggest that it is a DNA molecule composed of approximately 60 nucleotides, or 30 base pairs. However, an RNA molecule has not been rigorously excluded.

This report provides the first evidence for a negative endogenous regulator of topo I, and suggests that inhibitory regulation by physiologic concentrations of zinc may also occur. These mechanisms may be responsible for determining the ratio of active to inactive enzyme in nuclei.

They may also influence the DNA binding specificity of the enzyme, and therefore its activating effects on specific gene clusters, including the dermal collagen genes (6).

Modulators of topo I are also of potential therapeutic interest. We have found that a pharmacologic inhibitor of topo I, the alkaloid camptothecin selectively reduces both procollagen mRNA levels and collagen synthesis in fibroblasts (37). Camptothecin is used as an anti-cancer in China and is undergoing pre-clinical cancer trials in the U.S. (38-40). Given that zinc is also a topo I inhibitor, but acting directly in the enzyme (this report) rather than at the level of topo I-DNA complexes (41), the potential exists for synergy with camptothecin in reducing collagen production in scleroderma.

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