Degradation of DNA Topoisomerase I by a Novel Trypsin-like Serine Protease in Proliferating Human T Lymphocytes*

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DNA topoisomerase I (Topo I) contributes to various important biological functions, and its activity is therefore likely regulated in response to different physiological conditions. Increases in both the synthesis and degradation of Topo I were previously shown to accompany phytohemagglutinin stimulation of proliferation in human peripheral T lymphocytes. The mechanism of this degradation of Topo I has now been investigated with both in vivo and in vitro assays. The activity of a nuclear protease that specifically degrades Topo I was induced in proliferating T lymphocytes. The full-length Topo I protein (100 kDa) was sequentially degraded to 97- and 82-kDa fragments both in vivo and in vitro. The initial site of proteolytic cleavage was mapped to the NH2-terminal region of the enzyme. The degradation of Topo I in vitro was inhibited by aprotinin or soybean trypsin inhibitor, suggesting that the enzyme responsible is a trypsin-like serine protease. Furthermore, Topo I degradation by this protease was Mg2+-dependent. The Topo I-specific protease activity induced during T lymphocytes proliferation was not detected in Jurkat (human T cell leukemia) cells and various other tested human cancer cell lines, possibly explaining why the abundance of Topo I is increased in tumor cells.

The superhelical state of DNA is an important determinant of DNA structure and function. The enzyme DNA topoisomerase I (Topo I) regulates DNA topology and is evolutionarily conserved in both mode of action and amino acid sequence from yeast to mammals. Topo I relaxes DNA supercoils by cleaving a single strand of duplex DNA and passing the complementary DNA strand through the cleaved strand before religation (1).

Biochemical and genetic studies have shown that Topo I plays important roles in DNA replication (2, 3), RNA transcription (4–6), DNA recombination (7–10), chromosome condensation (11–13), and the maintenance of genomic stability (1, 14). During DNA replication, Topo I removes the positive supercoils that accumulate in the unreplicated portion of topologically restrained DNA as a result of the unwinding of the double helix. Topo I also acts as a swivel to release the torsional strain in DNA during RNA transcription. The enzyme contributes to illegitimate recombination between a cleavage complex and an exogenous DNA strand bearing a 5’-hydroxyl end. Moreover, Topo I regulates the initiation of transcription through direct interaction with transcription factors (15–17), and it catalyzes the phosphorylation of SR proteins, which are essential for RNA splicing (18).

Because Topo I participates in multiple biological functions, its expression is likely strictly regulated under various physiological conditions. Indeed, deregulation of Topo I results in fatal defects in cells. Thus, overexpression of yeast Topo I in Saccharomyces cerevisiae resulted in a 6–12-fold increase in the frequency of illegitimate recombination (19). Overexpression of human or yeast Topo I in Escherichia coli or of mammalian Topo I in mammalian cells resulted in cell death (20–22). The abundance of human Topo I protein is increased in tumors of the colon (23, 24), ovary (25), prostate (24), lung (26), esophagus (27), or acute myelogenous and acute lymphocytic leukemia (28), compared with that in the corresponding normal tissues. These observations also suggest that it is important for cells to maintain a normal level of Topo I protein.

The cellular concentration of a protein is determined by both its rate of synthesis and its rate of degradation. Control of protein stability is becoming a critical point for modulating gene expression, and the degradation of various proteins is known to be regulated. Thus, in eukaryotes, mitotic cyclins, G1 cyclin, and Cdk inhibitors are degraded at specific times during the cell cycle (29); proteolysis of p53 and e-Jun contributes to the control of cell growth and proliferation (29); a plant phytochrome is degraded after exposure to red light (30); and the rate of degradation of several important biosynthetic enzymes is increased in the presence of their end products (31, 32). In prokaryotes, the heat shock sigma factor, HtpR, is transiently stabilized in response to an increase in temperature (33). However, little is known about regulation of the stability of Topo I protein.

We have previously shown that PHA-induced T cell proliferation is accompanied by increases in both the abundance of Topo I mRNA and the synthesis of Topo I protein. However, we also detected a concomitant rapid degradation of Topo I under these same conditions (34). In the present study, we examined the mechanism of this rapid degradation of Topo I protein in PHA-stimulated proliferating T cells. We demonstrate that the degradation of Topo I is mediated by a nuclear trypsin-like serine protease and that the initial site of cleavage by this protease is located in the NH2-terminal region of Topo I. Our results also suggest that the abundance of Topo I is increased in tumor cells as a result of lacking the trypsin-like serine protease activity in the nucleus of these cells.

**EXPERIMENTAL PROCEDURES**

Materials—Lactacystin was kindly provided by Dr. E. J. Corey (Department of Chemistry, Harvard University). Aprotinin, leupeptin, pep-
lymphocytes were stimulated into proliferation by incubation in the E. coli Nonidet P-40, 0.5 mM DTT) to lyse the nuclei, the mixture was incubated for an additional 20 min with alkaline phosphatase-conjugated goat antibodies to rabbit IgG for immunoblot analysis. Rabbit antibodies to glutathione S-transferase (GST) were from Santa Cruz Biotechnology, and horseradish peroxidase-conjugated goat antibodies to rabbit IgG were from Southern Biotechnology Associates. All other chemicals were of analytical grade and from Sigma.

Cells and Culture Conditions—Human blood was obtained from healthy donors and mixed with heparin sulfate (20 units/ml). Lymphocytes were isolated from the heparinized blood by density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech) and were cultured at a density of 2 × 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 IU/ml), streptomycin (50 μg/ml), and 1 mM glutamine. The peripheral resting T lymphocytes were stimulated into proliferation by incubation in the presence of PHA (100 μg/ml) (Wellcome Diagnostics) at 37 °C. Jurkat cells (human T cell leukemia cell line), A2780 cells (human ovarian cell line), U937 cells (human monoblastic cell line), HL60 cells (human leukemia cell line), K562 cells (human erythroleukemia cell line), and EpPept-2 cells (human Epstein-Barr virus-transformed B lymphocytes) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. H460 cells (human non-small cell lung carcinoma cell line), A431 cells (human epidermoid cell line), Saos-2 cells (human osteosarcoma cell line), KB cells (human epidermoid carcinoma cell line), HepG2 cells (human hepatoblastoma cell line), and HeLa cells (human cervical carcinoma cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All cells were maintained under an atmosphere of 5% CO₂ and 95% air at 37 °C.

Expression Constructs and in Vitro Translation—Human Topo I cDNA was subcloned into the pET-22b plasmid (Novagen), and the resulting construct was subjected to in vitro transcription and translation with TNT wheat germ extract (Promega) in the presence of [35S]methionine (Amersham Pharmacia Biotech). The translation products were used directly in the in vitro assay of Topo I degradation (see below). Regions of human Topo I cDNA, generated either by the polymerase chain reaction or by restriction enzyme digestion, were also subcloned into the plasmid pGEX-KG (P-L Biochemicals) for expression of the encoded Topo I fragments as GST fusion proteins. Some of the resulting constructs (GST, GTOPI-4, GTOPI-6, and GTOPI-7) were subjected to in vitro transcription and translation with an in vitro transcription and translation kit (Promega) in the presence of [35S]methionine. The other GST-Topo I constructs were introduced into E. coli BL21 (DE3), and expression of the fusion proteins was induced by isopropyl-β-D-thiogalactopyranoside. The fusion proteins were purified with the use of glutathione-sepharose affinity columns. Both the fusion proteins produced by in vitro translation, and those purified with the affinity columns were used for the in vitro assay of Topo I degradation.

Preparation of Cytosolic and Nuclear Extracts—Cells were fractionated according to a modified version of the method of Dyer and Herzog (35). Cells (1 × 10^7) were collected by centrifugation at 200 × g for 5 min at 4 °C, washed with phosphate-buffered saline, and lysed by suspension in 100 μl of sucrose buffer 1 (0.32 M sucrose, 3 mM CaCl₂, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.9, 0.7 mM KCl, 15 mM MgCl₂, and centrifuged at 12,000 × g for 5 min at 4 °C. The resulting supernatant (cytosolic extract) was transferred to a fresh tube, mixed with 0.22 volume of 5× cytoplasmic extraction buffer (150 mM Hepes-NaOH, pH 7.9, 0.7 mM KCl, 15 mM MgCl₂), and centrifuged at 12,000 × g for 15 min at 4 °C. The resulting supernatant (cytosolic extract) was transferred to a fresh tube, into which sodium dodecyl sulfate was added to a final concentration of 25% (v/v), and the mixture was divided into portions and stored at -70 °C.

RESULTS

Induction of Nuclear Protease Activity Specific for Topo I by PHA in Peripheral T Lymphocytes—Our previous observation that Topo I is degraded rapidly during PHA stimulation of T lymphocyte proliferation (34) led us to propose that PHA may induce a protease activity that is responsible for this degradation. To investigate this proposal, we first harvested human T lymphocytes at various times after PHA stimulation and examined the degradation of Topo I by immunoblot analysis. The amount of Topo I gradually increased with time of exposure to PHA, and a partial degradation of Topo I into two smaller immunoreactive fragments was first detected 12 h after PHA stimulation and increased thereafter (Fig. 1), indicating that a protease activity was indeed induced in the proliferating T cells.

FIG. 1. Proteolysis of Topo I in PHA-stimulated human T lymphocytes. T cells were incubated with PHA for the indicated times, harvested, and resuspended in lysis buffer. Cell lysates (100 μg of protein) were subjected to immunoblot analysis with antisera to human Topo I. The positions of Topo I and of the two predominant degradation products are indicated.

In Vitro Assay of Topo I Degradation—5 μl of the [35S]methionine-labeled in vitro translated proteins or 250 ng of the purified GST-Topo I fusion proteins were incubated for various times at 37 °C in a final volume of 30 μl with nuclear or cytosolic extracts (10 μg of protein) isolated from proliferating (resting T lymphocytes stimulated with PHA for 72 h at 37 °C) or resting T lymphocytes in the incubation buffer (20 mM Hepes-NaOH, pH 8.0). After the addition of Laemmli sample buffer, the mixture was boiled for 5 min and then fractionated by SDS-polyacrylamide gel electrophoresis on a 10 or 12.5% gel. The gel was subjected to fluorography with 2,5-diphenyloxazol to detect the in vitro translated [35S]-labeled proteins. The degradation of the purified GST fusion proteins was detected by immunoblot analysis.

Immunoblot Analysis—Various times after stimulation with PHA, cells were harvested and lysed in an ice-cold solution containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μM aprotinin, 50 μM leupeptin, 30 mM sodium pyrophosphate, 50 mM NaF, 150 mM NaCl, 0.1% Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EDTA, and 50 mM Tris-HCl, pH 8.0. The mixture was incubated on a rotary platform at 4 °C for 20 min and centrifuged at 26,000 × g for 30 min at 4 °C. A portion (100 μg of protein) of each cell lysate was mixed with Laemmli sample buffer, boiled for 5 min, and fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel. The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore), which was then incubated for 2 h at room temperature in TBS buffer (125 mM NaCl, 25 mM Tris-HCl, pH 8.0) containing 1.5% (w/v) nonfat dried milk. The membrane was then incubated for 90 min with rabbit antisera to human DNA Topo I (1:5,000 dilution) (34) in TBST buffer (125 mM NaCl, 0.05% (v/v) Tween 20, 25 mM Tris-HCl, pH 8.0), washed three times with TBST buffer, and incubated with peroxidase-conjugated goat antibodies to GST and horseradish peroxidase-conjugated goat antibodies to rabbit IgG for 90 min each. The separated proteins were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

In vitro cleavage of GST-Topo I fusion proteins (GTOPI-1, -2, -3, -5, -8, and -9) was detected by immunoblot analysis with rabbit antibodies to GST and horseradish peroxidase-conjugated goat antibodies to rabbit IgG; immune complexes were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

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To characterize the proteolytic activity responsible for the degradation of Topo I, we developed an *in vitro* degradation assay. We produced $[^{35}S]$methionine-labeled human Topo I by *in vitro* transcription and translation and incubated the labeled protein with nuclear or cytosolic extracts isolated from either resting or proliferating T cells. The $[^{35}S]$-labeled Topo I (100 kDa) was degraded in a time-dependent manner in the presence of cytosolic or nuclear extracts from either resting (Fig. 2A) or proliferating (Fig. 3A) T cells. However, only nuclear extract from proliferating T cells generated a pattern of Topo I degradation similar to that observed in intact cells (Figs. 1 and 3A), characterized by two predominant proteolytic intermediates of 97 and 82 kDa that appeared to be produced sequentially. Whereas a mixture of protease inhibitors (aprotinin (5.8 μM), leupeptin (42 μM), pepstatin (1.46 μM), and PMSF (574 μM)) inhibited the degradation of Topo I *in vivo* by nuclear or cytosolic extracts from resting T cells (Fig. 2B) or by cytosolic extract of proliferating T cells (Fig. 3B), these inhibitors had no effect on Topo I degradation by nuclear extract of proliferating T cells (Fig. 3B), suggesting that this latter proteolysis is specific.

The 82-kDa processing intermediate was more prominent in the *in vitro* assay of Topo I degradation by nuclear extract of proliferating T cells (Fig. 3) than in the *in vivo* assay of Topo I degradation (Fig. 1). To investigate whether this difference might be due to an effect of temperature, given that the *in vivo* assay was performed at 4 °C and the *in vitro* assay was performed at 37 °C, we compared the patterns of *in vitro* degradation of Topo I at 37, 25, 15, and 4 °C. Although Topo I was degraded at all tested temperatures, the rate of proteolysis increased with increasing temperature, and the 82-kDa product was less evident at 4 °C than at 37 °C (Fig. 4). The *in vitro* degradation pattern at 4 °C was thus most similar to the *in vivo* pattern, suggesting that the degradation of Topo I into 97- and 82-kDa fragments is mediated by the same proteolytic activity both *in vivo* and *in vitro*.

To determine whether this proteolytic activity degrades other proteins, we incubated two nuclear proteins, the Cdk inhibitor p27 and the tumor suppressor p53 (Fig. 5), as well as bacterial GST (see Fig. 11A) with nuclear extract of proliferating T lymphocytes. None of these three proteins was degraded under conditions similar to those of the Topo I degradation assay. The protease activity thus appears to be relatively specific for Topo I.

**Characterization of the Proteolytic Activity Responsible for the Degradation of Topo I**—Protein turnover in mammalian cells has been shown to be mediated by three distinct pathways (36): the ubiquitin-26 S proteasome pathway, which requires ATP, and the lysosomal and Ca$^{2+}$-dependent pathways, which are ATP-independent. To determine whether Topo I is degraded by the ubiquitin-26 S proteasome pathway, we therefore examined the effects of the ATP analog ATP-γ-S, of depletion of ATP by apyrase, and of the 26 S proteasome inhibitors hemin and lactacystin on the degradation of Topo I by nuclear extract of proliferating T cells. The degradation of the 97-kDa Topo I fragment to the 82-kDa fragment was partially inhibited by 1 mM ATP-γ-S (Fig. 6A), and that of the full-length protein to the 97-kDa fragment was partially blocked at 10 mM ATP-γ-S and was completely inhibited at 40 mM ATP-γ-S (Fig. 6B). These data thus suggested that the degradation of Topo I may be ATP-dependent. However, depletion of ATP by apyrase failed to block Topo I degradation (Fig. 7A). To resolve these apparently inconsistent results, we examined the effect of ATP on Topo I proteolysis. The degradation of Topo I was inhibited by ATP in a concentration-dependent manner (Fig. 6C), similar to the effect of ATP-γ-S (Fig. 6B). As a control, we examined the effect of cAMP; this nucleotide had no effect on the degradation of Topo I (data not shown).

We have previously shown that the binding of ATP to Topo I results in a conformational change in the enzyme, whereas cAMP has no such effect (37). Thus, the inhibition of Topo I degradation by ATP or ATP-γ-S may be attributable to such a conformational change that renders the enzyme resistant to protease digestion. The conformation of Topo I may thus be a

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**Table 1.**

| RCE:  | - | + | + | - | - | - |
| RNE:  | - | - | - | - | + | + | + |
| **Time:** | 5 | 1 | 3 | 5 | 1 | 3 | 5 |

**Figure 2.** Degradation of Topo I by cytosolic or nuclear extracts of resting T lymphocytes *in vitro*. $[^{35}S]$Methionine-labeled Topo I was incubated for the indicated times at 37 °C with cytosolic (RCE) or nuclear (RNE) extracts of resting T cells either in the absence (A) or presence (B) of a mixture of protease inhibitors (aprotinin (5.8 μM), leupeptin (42 μM), pepstatin (1.46 μM), and PMSF (574 μM)). The reaction mixture was then analyzed by electrophoresis and fluorography. The *uppermost labeled band* corresponds to full-length Topo I.

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The conformation of Topo I may thus be a
determining factor of the stability of the protein. However, we also cannot rule out the possibility that the inhibitory effect of ATP or ATP-γ-S on the Topo I proteolysis is due to a titration of Mg\textsuperscript{2+}, because Mg\textsuperscript{2+} is required for Topo I proteolysis (see Fig. 9B). Our data indicate that ATP is not required for the proteolysis of Topo I. Neither lactacystin (Fig. 7B) nor hemin (data not shown) inhibited the degradation of Topo I, further indicating that Topo I proteolysis is not mediated by the ATP-dependent 26 S proteasome pathway in proliferating T lymphocytes.

The effects of E64 as well as of calpain I inhibitor and calpain II inhibitor on Topo I degradation were examined to determine the role of the lysosomal and Ca\textsuperscript{2+}-dependent proteolysis systems, respectively, in this phenomenon. None of these three inhibitors blocked the proteolysis of Topo I (data not shown). Together, our data suggest that the degradation of Topo I is not mediated by the ubiquitin-dependent 26 S proteasome, lysosomal, or Ca\textsuperscript{2+}-dependent proteolytic pathways.

Role of a Trypsin-like Serine Protease in the Degradation of Topo I—Proteases can be classified into four types on the basis of their active sites: serine proteases, cysteine proteases, metalloproteases, and aspartic proteases. Proteases of each type are inhibited by corresponding specific blockers. To characterize the type of protease responsible for the degradation of Topo I, we examined the effects of various protease inhibitors, including aprotinin, PMSF, pepstatin, leupeptin, soybean trypsin inhibitor, EDTA (Fig. 8), N-ethylmaleimide, N-a-tosyl-l-lysine chloromethyl ketone, tosyl-l-phenylalanine chloromethyl ketone, chymostatin, and caspase inhibitors (data not shown), on Topo I degradation by nuclear extract of proliferating T cells. Of these various agents, only EDTA, aprotinin (trypsin inhibitor, pancreas type, from bovine lung), and soybean trypsin inhibitor exhibited an inhibitory effect on Topo I degradation. The degradation of the 97-kDa fragment of Topo I to the 82-kDa fragment was inhibited by aprotinin at concentrations of 50 and 167 μM (Fig. 8B); however, aprotinin failed to block the degradation of the full-length protein to the 97-kDa intermediate. Soybean trypsin inhibitor blocked the degradation of full-length Topo I in a concentration-dependent manner, with complete inhibition of proteolysis apparent at a concentration of 150 μM (Fig. 8C). Taken together, these results suggest that the protease responsible for Topo I degradation is an EDTA-sensitive, trypsin-like serine protease.

Requirement of Mg\textsuperscript{2+} for Degradation of Topo I—The degradation of the 97-kDa fragment of Topo I to the 82-kDa fragment was inhibited by 1 mM EDTA (Fig. 8A). Furthermore, degradation of the full-length enzyme to the 97-kDa intermediate was inhibited in the presence of 2 mM EDTA (Fig. 9A). These observations suggested that Mg\textsuperscript{2+} or Ca\textsuperscript{2+} might be required for Topo I proteolysis. We therefore investigated the effect of the Ca\textsuperscript{2+}-specific chelator EGTA and addition of Ca\textsuperscript{2+} on this process. EGTA had no effect on the degradation of Topo I at concentrations up to 2 mM (Fig. 9A); however, addition of various concentrations of Ca\textsuperscript{2+} also did not stimulate proteolysis of Topo I (data not shown), indicating that Ca\textsuperscript{2+} is dispensable for proteolysis of Topo I. We further investigated the role of Mg\textsuperscript{2+}...
in Topo I degradation by examining the effect of addition of various concentrations of Mg\(^{2+}\) to the assay mixture in the presence of 2 mM EDTA. The addition of Mg\(^{2+}\) reversed the inhibitory effect of EDTA on the degradation of Topo I in a concentration-dependent manner (Fig. 9B), indicating that Mg\(^{2+}\) is required for Topo I proteolysis.

**Mapping of the Site of Proteolytic Cleavage in Topo I**—To localize the site of proteolytic cleavage in Topo I, we constructed recombinant GST fusion proteins containing various fragments of Topo I (Fig. 10). The fusion proteins were either expressed in *E. coli* and purified by glutathione affinity chromatography or translated in *vitro*, and their degradation was then examined with the *in vitro* assay. Only the GTOPI-1 fusion protein (amino acids 1–138 of Topo I) was cleaved by the nuclear extract of proliferating T lymphocytes (Fig. 11, A–D). To assess whether the degradation of GTOPI-1 and that of full-length Topo I are likely mediated by the same protease, we examined the effect of soybean trypsin inhibitor on the proteolytic cleavage of GTOPI-1. The degradation of GTOPI-1 was inhibited by soybean trypsin inhibitor in a dose-dependent manner (Fig. 11E), suggesting that the proteolysis of GTOPI-1 and that of full-length Topo I are mediated by the same trypsin-like serine protease. The fusion protein data indicate that the initial site of Topo I cleavage, which reduces the molecular size of the protein from 100 to 97 kDa, is located within the NH\(_2\)-terminal 50 residues of the protein.

**Lack of the Trypsin-like Serine Protease Activity in Human Tumor Cell Lines and Epstein-Barr virus-transformed B Cells**—We next investigated whether the protease activity responsible for the degradation of Topo I in normal proliferating T lymphocytes is also expressed in human transformed lymphocyte and other cell lines. We prepared cytosolic and nuclear extracts of A2780, Jurkat, HeLa, H460, KB, Saos-2, A431, HepG2, K562, U937, HL60, and phorbol 12-myristate 13-acetate-treated HL60 cells as well as of Epstein-Barr virus-transformed B lymphocytes. These extracts were then examined for their ability to cleave full-length Topo I in *vitro*. Full-length Topo I was sequentially processed to the 97- and 82-kDa fragments by nuclear extract of proliferating T lymphocytes (Fig. 12A), whereas Topo I remained intact during incubation with either nuclear extract (Fig. 12A) or cytosolic extract (data not shown) of Jurkat cells. The trypsin-like serine protease activity was also not detectable in nuclear extract of the A2780 ovarian cancer cell line (Fig. 12B) or in the extracts of Epstein-Barr virus-transformed B lymphocytes or any of the other tested cancer cell lines (data not shown). The activity of this protease

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**Fig. 6. Effects of ATP-γ-S and ATP on the degradation of Topo I in *vitro***. [\(^{35}\)S]Methionine-labeled Topo I was incubated for 5 min at 37 °C in the presence of the indicated concentrations of ATP-γ-S (A and B) or ATP (C) and in the absence or presence of nuclear extract of proliferating T cells. The degradation of Topo I was then analysed as described in the legend to Fig. 2. The leftmost lane in A contains molecular size standards. PNE, nuclear extracts of proliferating T lymphocytes.

**Fig. 7. Effects of apyrase and lactacystin on the degradation of Topo I in *vitro***. A, [\(^{35}\)S]methionine-labeled Topo I and nuclear extract of proliferating T lymphocytes, each of which had been pretreated or not with apyrase, were incubated in the indicated combinations with a mixture of protease inhibitors (aprotinin, leupeptin, pepstatin, and PMSF) for 5 min at 37 °C. B, [\(^{35}\)S]methionine-labeled Topo I was incubated for 5 min at 37 °C in the presence of the indicated concentrations of lactacystin and in the absence or presence of nuclear extract of proliferating T cells. Topo I degradation was analyzed as described in the legend to Fig. 2. PNE, nuclear extracts of proliferating T lymphocytes.
thus appears to be induced when resting T lymphocytes enter proliferation rather than being expressed in actively cycling cells.

**DISCUSSION**

Regulation of protein stability has been revealed to be important in cell cycle progression, signal transduction, transcription factor activity, tumor suppression, and oncogenesis. Abnormalities in protein degradation often result in disruption of normal physiological status and underlie various diseases (38–40). However, little has been known of the stability or turnover of human Topo I (41).

We previously showed that the abundance of Topo I is regulated when human peripheral T lymphocytes stimulated with PHA. Topo I protein was thus shown to be synthesized in large amounts but also rapidly degraded when resting T lymphocytes are induced to proliferate (34). We have now established an *in vitro* assay of Topo I proteolysis that appears to mimic the degradation of the protein in intact cells. We have also characterized a novel trypsin-like serine protease that appears to mediate the degradation of human Topo I in PHA-stimulated T

![FIG. 8. Effects of various protease inhibitors on the degradation of Topo I in *vitro*.](image1)

![FIG. 9. Effect of Mg²⁺ on the degradation of Topo I in *vitro*.](image2)

![FIG. 10. Schematic representation of GST-Topo I fusion proteins and summary of *in vitro* cleavage data.](image3)
lymphocytes. This protease is localized to the nucleus and degrades Topo I sequentially into 97- and 82-kDa fragments; the latter undergoes further degradation in vitro with prolonged incubation times (Fig. 12A).

Topo I exists in cells as a phosphorylated protein under various physiological conditions (42–45). To rule out the possibility that the 97-kDa intermediate is actually a less phosphorylated or unphosphorylated form of full-length Topo I, we investigated the effect of addition of a phosphatase inhibitor (aprotinin, leupeptin, pepstatin, and PMSF) to the degradation assay mixture; the inhibitor had no effect on the pattern of Topo I degradation (data not shown). Furthermore, the generation of the 97-kDa product in vitro was inhibited in a concentration-dependent manner by soybean trypsin inhibitor. The 97-kDa intermediate thus indeed appears to be a product of Topo I proteolysis. We also showed that Mg²⁺ is required for the degradation of Topo I in vitro. However, it remains unclear whether Mg²⁺ acts as a cofactor for the protease or is required for Topo I itself. Although a requirement of Mg²⁺ for proteolysis is rare, several precedents exist. For example, Mg²⁺ is required both for the proteolytic function of amidopeptidase (46–48) and for the ubiquitin-dependent 26 S proteasome degradative pathway (29).

Desai et al. (41) showed that Topo I is degraded by the ubiquitin-dependent 26 S proteasome pathway in Chinese hamster ovary cells and that Topo I degradation by this pathway is stimulated by camptothecin. Fu et al. (49) also showed that the camptothecin-induced degradation of Topo I in KB cells is mediated by the ubiquitin-dependent 26 S proteasome. However, in our study, apyrase, lactacystin, and hemin did not affect the degradation of Topo I in vitro. The possibility that both the trypsin-like serine protease and the ubiquitin-dependent 26 S proteasome pathway are responsible for the degradation of Topo I in our system appears to be excluded by the observation that the abundance of Topo I did not decrease with incubation time (from 5 to 30 min) after inhibition of the trypsin-like serine protease with 200 μM soybean trypsin inhibitor (data not shown). Thus, the degradation of Topo I may be mediated predominantly by the trypsin-like serine protease in normal proliferating T cells, whereas Topo I proteolysis is mediated by the ubiquitin-dependent 26 S proteasome in cells...
with camptothecin-induced DNA damage.

No intracellular protease with the characteristics (protease inhibitor sensitivity and subcellular localization) of the trypsin-like protease detected in the present study has been previously described. Hirschhorn et al. (50) suggested that a trypsin-like protease activity is required for the increase in RNA transcription in the nucleus when resting T lymphocytes stimulated with PHA. It remains to be determined whether this protease is identical to that described in the present study. Our results also appear to differ from those of Grayzel et al. (51), who did not detect an increase in broad spectrum proteolytic activity (measured with casein and hemoglobin as substrates) in response to exposure of resting T lymphocytes to PHA. This apparent discrepancy may be due to the difference in substrates used in the respective assays.

The susceptibility of a protein to proteolysis can be regulated by phosphorylation (29), conformation (52), and the existence of intrinsic destabilizing sequences (29). In the present study, the addition of various types of kinase inhibitors or of calf intestinal phosphatase to the nuclear extract of proliferating T cells had no effect on the degradation of Topo I by this extract (data not shown), suggesting that the phosphorylation state of Topo I does not affect its susceptibility to proteolysis. In addition, recombinant Topo I produced with the use of a baculovirus expression system is a phosphoprotein, and the purified phosphoprotein was also degraded by the trypsin-like serine protease in our in vitro assay (data not shown). We previously showed that Topo I exists in at least two distinct conformations; one conformation binds ATP and catalyzes the phosphorylation of SR proteins, whereas the other form binds to and relaxes superhelical DNA (37). Our present observation that Topo I degradation was inhibited by ATP and ATP-γ-S thus suggests that the conformation of ATP-bound Topo I is resistant to digestion by the trypsin-like serine protease, whereas the conformation of DNA-bound Topo I is sensitive to the action of this protease. However, we cannot rule out the possibility that this ATP inhibition is through the titration of the Mg$^{2+}$ ions.

The site of initial cleavage in Topo I was mapped to the NH$_2$-terminal 50 amino acids of the protein. Cleavage at this site is likely responsible for initiating proteolysis, given that the 97-kDa product appeared to be an essential intermediate for further degradation. We were not able to map the second cleavage site, possibly because cleavage at the first site by the trypsin-like serine protease results in a structural change in the 97-kDa intermediate that renders the second site susceptible to attack by the protease. On the basis of this hypothesis, given that, of the various GST-Topo I fusion proteins studied, only GTOPI-1 possesses the first cutting site, these constructs would not have been informative with regard to the localization of the second cleavage site.

Studies with human (53) and yeast (54) Topo I have revealed that the NH$_2$-terminal domains of these enzymes are dispensable for catalytic activity but that they may play an important role in the in vivo function of Topo I. Shaw and Hsieh (55) recently showed that the hydrophilic NH$_2$-terminal domain of Drosophila Topo I targets to transcriptionally active loci. It is therefore possible that human Topo I has a similar function. Interestingly, all of the proteolytic cleavage sites of human Topo I described to date are located in the NH$_2$-terminal domain of the protein. Thus, in addition to the results of the present study, the NH$_2$-terminal domain of Topo I has previously been shown to be susceptible to proteolysis (56), and Topo I degradation induced by apoptosis was shown to occur by cleavage at the NH$_2$ terminus (57). Some cues were provided to explain why all the proteolytic cleavage sites occurred on the NH$_2$-terminal domain of Topo I. The NH$_2$-terminal portion of Topo I forms an extensive structure (56) that would appear inherently susceptible to protease attack. Furthermore, removal of the NH$_2$-terminal domain may facilitate proteolytic attack on the remaining portion of Topo I. Functionally, removal of the NH$_2$-terminal domain may inactivate the biological function of Topo I. An example is Drosophila Topo I that deletion of NH$_2$ terminus impairs its targeting to actively transcribed gene and blocks the involvement of Topo I in RNA transcription (55). Therefore, the activity of Topo I may be finely tuned by scissoring off its NH$_2$ terminus immediately to inactivate its biological functions and to facilitate the proteolysis of its residual portion.

The rapid increase in the abundance of Topo I apparent on stimulation of proliferation in peripheral T cells likely ensures the effective execution of the associated DNA replication and gene transcription. However, such a rapid increase in Topo I abundance also confers a potential risk of genomic instability. Thus, overexpression of Topo I in yeast promotes illegitimate recombination (19). Illegitimate recombination events are usually associated with chromosomal aberrations, which, in turn, are implicated in carcinogenesis and many heritable diseases. In addition, the transcriptional activity of other genes may be affected by changing in Topo I abundance (58). This change could be involved in the activation of oncogenes, loss expression of tumor suppressor genes, or deregulation of genes whose products contribute to recombination. Therefore, it is likely that Topo I is removed by proteolysis immediately after its abundance has increased to meet the physiological requirements of the cell.

The abundance of Topo I in cancer cells has been shown to be an average of 14-fold greater than that in the corresponding mucosal cells of individuals with colon cancer (23, 24). A similar phenomenon has also been detected for other cancers (24–28). However, the mechanism responsible for this difference remains unclear. In the present study, we have shown that a novel trypsin-like serine protease activity that mediates the degradation of Topo I is induced in normal proliferating T cells, but this activity is not detectable in Jurkat cells and other various cancer cell lines. The lack of activity of this trypsin-like serine protease in nuclear extracts of these cells may thus explain why the abundance of Topo I is increased in cancer cells.

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