Apoptotic topoisomerase I-DNA complexes induced by staurosporine-mediated oxygen radicals

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Running title: Apoptotic Top1-DNA complexes induced by staurosporine
Summary

Topoisomerase I (Top1), an abundant nuclear enzyme expressed throughout the cell cycle, relaxes DNA supercoiling by forming transient covalent DNA cleavage complexes. We show here that staurosporine, an ubiquitous inducer of apoptosis in mammalian cells, stabilizes cellular Top1 cleavage complexes. These complexes are formed indirectly as staurosporine cannot induce Top1 cleavage complexes in normal DNA with recombinant Top1 or nuclear extract from normal cells. In treated cells, staurosporine produces oxidative DNA lesions and generates reactive oxygen species (ROS). Quenching of these ROS by the antioxidant N-acetyl-L-cysteine or inhibition of the mitochondrial-dependent production of ROS by the caspase inhibitor benzyloxycarbonyl-VAD prevents staurosporine-induced Top1 cleavage complexes. Downregulation of Top1 by small interfering RNA decreases staurosporine-induced apoptotic DNA fragmentation. We propose that Top1 cleavage complexes resulting from oxidative DNA lesions generated by ROS in staurosporine-treated cells contribute to the full apoptotic response.

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Introduction

We previously reported in a meeting abstract that staurosporine induces Top1 cleavage complexes as mammalian cells undergo apoptosis (1). However, at that time, the mechanism of formation of these complexes and their role in apoptosis remained incompletely investigated. Apoptosis, the cell’s death program, plays a crucial role for development and for adult homeostasis (2). Staurosporine, an alkaloid kinase inhibitor, is widely used to study the mechanisms of cell death because of its unique ability to induce apoptosis in a wide variety of mammalian cells. Although the mechanism(s) by which staurosporine initiates apoptosis is still unclear, it is now apparent that staurosporine induces apoptosis through the mitochondrial pathway (3,4). Staurosporine increases the permeability of mitochondria outer membrane, thereby allowing the release of proteins normally located in the space between the inner and the outer mitochondrial membranes (5,6). Some of these molecules are involved in the activation of caspases (cytochrome c, Smac/DIABLO, HtrA2/Omi), and other in nuclear modifications (endonuclease G, AIF) (7). After cellular exposure to staurosporine, activated caspase-3 feeds back on permeabilized mitochondria, which further dissipates the mitochondrial transmembrane potential (Δψm) and induces the accumulation of intracellular reactive oxygen species (ROS) (8).

DNA topoisomerase I (Top1) is an ubiquitous and essential enzyme as it relaxes DNA supercoiling ahead of replication and transcription complexes (9-11). DNA relaxation is due to the induction of transient single-strand breaks, thereby allowing rotation of the DNA double helix around the intact phosphodiester bonds opposite to the
enzyme-mediated DNA cleavages. Once the DNA is relaxed, Top1 readily religates the break and regenerates intact duplex DNA. Under normal conditions, the covalent Top1-cleaved DNA intermediates, referred to as “cleavage complexes” are transient and remain at very low levels; the DNA religation (“closing”) step being much faster than the DNA cleavage (“nicking”) step. Top1 cleavage complexes can be schematically produced by two main mechanisms. First, Top1 cleavage complexes can be trapped by specific inhibitors such as camptothecin and its chemotherapeutic derivatives. These anticancer drugs specifically bind at the Top1-DNA interface and trap the cleavage complexes by preventing the DNA religation step (12-14). The second mechanism is related to DNA lesions that interfere with Top1’s DNA nicking-closing activities. These modifications include frequent lesions such as oxidized bases (e.g. 8-oxoguanine), abasic sites, mismatches and strand breaks (15-17).

Recently, Top1 cleavage complexes have been observed in cells undergoing apoptosis following treatment with arsenic trioxide (18) or UV irradiation (19). In the present study, we describe the staurosporine-induced apoptotic Top1 cleavage complexes, and report the mechanism of their production and functional relevance. We demonstrate that Top1 cleavage complexes are related to DNA modifications during staurosporine-induced apoptosis rather that resulting from a direct drug-Top1 interaction. Our results suggest that staurosporine-induced Top1 cleavage complexes form in response to oxidative DNA lesions generated by radical oxygen species (ROS) and caspase activation. Finally we provide evidence for the functional role of Top1-DNA complexes in chromatin fragmentation during staurosporine-induced apoptosis.
Experimental procedures

Drugs and chemical reagents

Staurosporine (STP) and N-acetyl-L-cysteine (NAC) were obtained from Sigma; camptothecin (CPT) was obtained from the Drug Synthesis and Chemistry Branch, NCI (Bethesda, MD); and the caspase peptide inhibitor benzyloxy carbonyl-Val-Ala-DL-Asp(OMe)-fluoromethylketone (Z-VAD-fmk) was from Bachem (Torrance, CA). Stock solutions for STP, CPT, and Z-VAD-fmk were prepared in dimethyl sulfoxide (Me₂SO) and stored at −20 °C. Further dilutions were made in culture medium just before use. The final concentration of Me₂SO in culture medium never exceeded 0.1% (vol/vol), which was non-toxic to the cells. NAC was diluted in culture medium prior to use. [2-¹⁴C]Thymidine was obtained from PerkinElmer Life Sciences. Apurinic/apyrimidinic endonuclease-1 (Ape1) was obtained from Sigma, topoisomerase I (Top1) and formamidopyrimidine-DNA glycosylase (Fpg) were prepared as described previously (17,20).

Cell Culture

The human leukemia (CEM, HL-60) and colon carcinoma (HCT116) cell lines (American Type Culture Collection, Manassas, VA) were culture in RPMI 1640 and Dulbecco’s modified Eagle’s medium, respectively. Culture mediums were supplemented with 10% (vol/vol) fetal bovine serum (Gemini Bio-Products, Woodland, CA) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). The murine leukemia cell line
P388 and its camptothecin-resistant, Top1-deficient subline, P388/CPT45 (21), were gifts from M. R. Mattern and R. K. Johnson (GlaxoSmithKline).

Detection of cellular Top1-DNA complexes.

Top1-DNA complexes were detected using the \textit{in vivo} complex of enzyme bioassay (22). Briefly, $10^6$ cells were lysed in 1% Sarkosyl and homogenized with a Dounce homogenizer. Cell lysate was layered on cesium chloride step gradients and centrifuged at 165,000 x $g$ for 20 h at 20 °C. Twenty fractions (0.5 ml each) were collected from the bottom, diluted into an equal volume of 25 mM potassium phosphate buffer, pH 6.6, and applied to polivinylidene difluoride membrane (Immobilon-P, Millipore, MA) by using a slot-blot vacuum manifold. Top1-DNA complexes were detected by immunoblotting using the C21 Top1 mouse monoclonal antibody (a kind gift from Dr. Yung-Chi Cheng, Yale University, New Haven, CT).

Top1 cleavage complexes with recombinant Top1 and nuclear extracts

3'-end-labeled DNA fragments were incubated with Top1 (17) or 0.35 M NaCl nuclear extracts (23). DNA fragments resulting from Top1 cleavage were separated by polyacrylamide gel electrophoresis and visualized by PhosphorImager (Amersham Biosciences).

Detection of oxidative DNA lesions

CEM cells were labeled with $[2^{-14}C]$thymidine (0.04 $\mu$Ci/ml) for 24 h, and chased for 12 h in radioisotope-free medium. After drug treatment, cells were permeabilized in
hypotonic buffer (10 mM Tris-HCl, pH 7.8, 70 mM NaCl, 1 mM EDTA, 1 mM DTT) containing 0.05% triton X-100 at 4 °C for 15 min by gentle mixing. Fpg (100 ng/100 µl reactions) was added for 30 min at 25 °C, and DNA breaks were analyzed by alkaline elution as previously described (24).

**In vitro chromatin digestion assay**

Nuclei were isolated from HL-60 cells as described previously (25). Nuclei (7 x 10⁶) were resuspended in STKM buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.0, 25 mM KCl, and 5 mM MgCl₂) and mixed with purified Ape1 (30 units) in the presence of 1 mM DTT. Reaction mixtures were incubated at 37 °C for 90 min. DNA was extracted by standard phenol/chloroform/isoamyl alcohol extraction procedures, and analyzed by 1.3% agarose gels electrophoresis.

**DNA fragmentation assays**

DNA fragmentation-related apoptosis was quantified using the previously reported filter elution assay (26). Cells were incubated with [2-¹⁴C]thymidine (0.02 µCi/ml) for 2 days, and chased overnight in radioisotope-free medium. After drug treatment, cells were loaded onto a protein-absorbing filter (Metricel® Membrane Filter, 0.8-µm pore size, 25 mm diameter; Pall Corp.), washed with phosphate-buffered saline, and lysed in 0.2% sodium Sarkosyl, 2 M NaCl, 0.04 M EDTA, pH 10.0. The filters were then washed with 0.02 M EDTA, pH 10.0. DNA was depurinated by incubation of filters in 1 M HCl at 65 °C and then released from the filters with 0.4 M NaOH at room temperature. Radioactivity was counted by liquid scintillation spectrometry in each fraction (wash,
lysis, EDTA wash, and filter). DNA fragmentation was measured as the fraction of disintegrations/min in the lysis fraction plus EDTA wash relative to the total intracellular disintegrations/min. For sub-G₁ analysis, DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (BD Biosciences). The numbers of cells with sub-G₁ DNA were determined with a CellQuest program (BD Biosciences).

**Caspase-3 activity**

Caspase-3 activity was measured as described (27). Cells were incubated in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) for 30 min at 4 °C and centrifuged (10,000 x g, 20 min, 4 °C). Twenty micrograms of protein from the resulting supernatant was incubated in buffer assay (100 mM HEPES, pH 7.0, 1 mM EDTA, 0.1% CHAPS, 10% glycerol, 20 mM dithiothreitol) in the presence of 100 µM of the fluorogenic peptide substrate Z-DEVD-AFC (Calbiochem). 7-Amino-4-trifluoromethylcoumarin (AFC) released from the substrate was excited at 400 nm to measure emission at 505 nm. Fluorescence was monitored continuously at 37 °C for 20 min in a dual luminescence fluorimeter (SPECTRAmax® GEMINI XS, Molecular Devices). Enzyme activities were determined as initial velocities expressed as relative intensity/min/mg.

**Immunoblotting**

Cells were lysed at 4 °C in buffer containing 1% SDS, 1 mM sodium vanadate, 10 mM Tris-HCl, pH 7.4, supplemented with protease inhibitors (Complete; Roche Diagnostics).
Viscosity of the samples was reduced by brief sonication, and 20 µg of protein (Bio-Rad Protein Assay; Bio-Rad) were incubated in loading buffer (125 mM Tris-HCl, pH 6.8, 10% -mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromophenol blue), separated by SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, MA). After blocking nonspecific binding sites for 1 h by 0.2% casein in TPBS (phosphate-buffered saline, Tween 20 0.1%), the membrane was incubated for 2 h with primary antibody. Antibodies used include rabbit antihuman poly(ADP-ribose)-polymerase (PARP; Roche) (1:5,000 dilution) and mouse antihuman Top1 (clone C21) (1:1,000 dilution). After three washes in TPBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:20,000 dilution) or anti-mouse (1:5,000 dilution) antibody (Amersham Biosciences) for 1 h and then washed three times in TPBS. Immunoblot was revealed using enhanced chemiluminescence detection kit (Pierce) by autoradiography.

**ROS detection**

Cells were exposed to 0.5 µM H$_2$DCF-DA (Molecular Probes) for 5 min and then examined by phase contrast and fluorescence microscopy. All photomicrographs were taken at equal magnification (20X) and exposure times. Quantification of DCF fluorescence was determined with the Adobe Photoshop program and expressed as relative fluorescence intensity/cell.
Top1 silencing by RNA Interference

Top1 was silenced in HCT116 cells by the transfection of U6 promoter-driven DNA vectors stably expressing siRNA hairpins targeting human Top1 (cDNA sequence: 5'-CTT GAC AGC CAA GGT ATT C-3') or a negative control sequence (cDNA sequence: 5'-GCG TCC TTT CCA CAA GAT A-3') as described (18).

Results

Staurosporine induces Top1-DNA complexes in cells undergoing apoptosis. The presence of Top1-DNA complexes in genomic DNA can be detected after cesium chloride gradient centrifugation and fractionation from tissue culture cells or tumor samples (22). In staurosporine-treated leukemia CEM cells, immunoblotting revealed the presence of Top1 in the DNA-containing fractions [fractions 7-10] (Fig. 1A). Unlike the Top1 inhibitor camptothecin, staurosporine was not able to generate Top1 cleavage complexes in normal DNA in the presence of recombinant human Top1 (Fig. 1B), indicating that staurosporine does not directly poison Top1. Cellular Top1-DNA complexes are therefore likely to be secondary to intracellular modifications induced by staurosporine.

Given that staurosporine is a potent inducer of apoptosis, we tested whether the cellular Top1-DNA complexes resulted from the engagement of apoptotic pathway(s). In CEM cells, staurosporine induces detectable Top1-DNA complexes after 3 h (Fig. 2A), concomitantly with DNA fragmentation (Fig. 2B), and activation of caspases, which was
Staurosporine induces apoptotic Top1-DNA complexes, p. 11 of 22

demonstrated by the ability of cell lysates to cleave DEVD-AFC, a fluorogenic peptide substrate for caspase-3 and closely related caspases (Fig. 2C, top panel). Caspases activation was further confirmed by the caspase-dependent cleavage of poly(ADP-ribose)-polymerase (PARP) (Fig. 2C, bottom panel). Staurosporine-induced Top1-DNA complexes were also observed in the human colon carcinoma HCT116 cells undergoing apoptosis (Fig. 2, D to F), and in human leukemia HL-60 cells (supplementary Fig. 2). Thus, the induction of Top1-DNA complexes by staurosporine can be observed in different human cell types as they undergo apoptosis.

**Staurosporine induces oxidative DNA lesions.** Because Top1 cleavage complexes can be produced by oxidative DNA lesions such as oxidized bases, abasic sites and strand breaks (15-17), we investigated the generation of such lesions by staurosporine. Permeabilized CEM cells were exposed to purified formamidopyrimidine DNA glycosylase (Fpg), an enzyme that converts oxidized purines (e.g., 8-oxoguanine) into DNA single-strand breaks (SSB) (20). Using the alkaline elution assay (24), we observed that Fpg induced SSB in staurosporine-treated cells (Fig. 3A), indicating the presence of oxidative base damage. We also tested base damage in staurosporine-treated cells using recombinant apurinic endonuclease 1 (Ape1) that cleaves DNA adjacent to abasic sites (28). As visualized by agarose gel electrophoresis, Ape1 induced chromatin fragmentation in nuclei from staurosporine-treated cells (Fig. 3B).

Nuclear extracts from staurosporine-treated cells failed to cleave a normal duplex oligonucleotide containing a canonical Top1 cleavage site (29) (Fig. 3C), indicating that the effect of staurosporine is not on Top1 itself.
**Inhibition of ROS prevents apoptotic Top1-DNA complexes.** To further investigate whether apoptotic Top1-DNA complexes result from oxidative DNA lesions, we used 2',7'-dichlorohydrofluorescein diacetate (H$_2$DCF-DA) to detect intracellular H$_2$O$_2$ and other peroxides (30). Fluorescence microscopy showed that staurosporine increased ROS levels in CEM cells (Fig. 4, A and B). Then, we investigated whether inhibition of ROS levels would affect the generation of Top1-DNA complexes. Treatment of CEM cells with the antioxidant N-acetyl-l-cysteine (NAC) together with staurosporine prevented both staurosporine-induced ROS production (Fig. 4, A and B) and Top1-DNA complexes (Fig. 4C).

Caspases have been recently involved in the production of ROS by staurosporine (8). Consistently, inhibition of caspases by the peptide Z-VAD-fmk prevented staurosporine-induced ROS production (Fig. 4, A and B). Under these conditions, Z-VAD-fmk also prevented the formation of Top1-DNA complexes (Fig. 4C). Altogether, these findings support the hypothesis linking oxidative DNA damage with Top1-DNA complexes during apoptosis.

**Apoptotic Top1-DNA complexes contribute to DNA fragmentation.** As shown in Fig. 5A, NAC prevents apoptotic DNA fragmentation in staurosporine-treated CEM cells. To further evaluate its involvement in chromatin fragmentation during apoptosis, Top1 was downregulated by small interfering RNA (siRNA) hairpins targeting human Top1 (Fig. 5B). Top1 silencing reduced staurosporine-induced DNA fragmentation in HCT116 cells (Fig. 5C). Similar results were observed with another siRNA hairpins sequence targeting
Top1 (supplementary Fig. 5). Apoptotic DNA fragmentation (Fig. 5D) and internucleosomal DNA laddering (Fig. 5E) were also reduced in P388/CPT45 cells deficient for Top1 (31). Together, these findings suggest the contribution of Top1 cleavage complexes to apoptotic DNA fragmentation.

Discussion

This study reports the formation of Top1 cleavage complexes by staurosporine, an ubiquitous inducers of apoptosis in mammalian cells. These apoptotic Top1 cleavage complexes were observed in three human cell lines (two leukemia [CEM, HL-60] and one colon carcinoma [HCT116] cell line) undergoing apoptosis. Staurosporine is a protein kinase inhibitor (32) that cannot induce Top1 cleavage complexes in normal DNA in the presence of recombinant Top1 or nuclear extracts from normal cells (Fig. 1 and 3). Thus, the mechanism of formation of the apoptotic Top1 complexes induced by staurosporine is indirect rather than resulting from drug-Top1 interaction.

We have observed that oxidative DNA lesions occur during staurosporine-induced apoptosis likely as a result of ROS production (Fig. 3 and 4), and we propose that staurosporine-induced apoptotic Top1 cleavage complexes result from these DNA modifications. Consistently, quenching of ROS with the antioxidant NAC prevents staurosporine-induced Top1 cleavage complexes (Fig. 4). Similarly, arsenic trioxide induces oxidative DNA lesions (33) and apoptotic Top1 cleavage complexes (18), suggesting that the ROS-dependent formation of oxidative DNA lesions is a common
mechanism for the trapping of Top1 cleavage complexes during apoptosis (34). In fact, cellular exposure to hydrogen peroxide (H$_2$O$_2$) was recently shown to induce Top1 cleavage complexes (35). Thus we propose that staurosporine induces the generation of ROS that damage DNA (oxidized bases, abasic sites), thereby generating Top1 cleavage complexes in apoptotic cells (Fig. 6). It is also possible that some of the DNA breaks produced by apoptotic nucleases such as DFF40/CAD (36), endonuclease G (37), or Ape1 (38) contribute to the trapping of Top1 cleavage complexes (15).

Cellular exposure to staurosporine induce mitochondrial outer membrane permeabilization (3), which is followed by the release of cytochrome c and the downstream activation of caspase-9 and caspase-3 (6). Activated caspase-3 was recently shown to feed back on permeabilized mitochondria and cleave the 75-kDa subunit (NDUFS1) of complex I, which leads to a disruption of the mitochondrial transmembrane potential ($\Delta$\text{m}) and the production of ROS (8). Mitochondrial production of ROS is likely to contribute to Top1 cleavage complexes formation since caspase inhibition by Z-VAD-fmk inhibits staurosporine-induced ROS and Top1 cleavage complexes (Fig. 4). Activation of caspases could therefore serve to generate ROS that lead to Top1 cleavage complexes during staurosporine-induced apoptosis (Fig. 6).

Apoptotic Top1 cleavage complexes probably contribute functionally to apoptosis as silencing of Top1 (Fig. 5B) reduces apoptotic DNA fragmentation (Fig 5C). Also, Top1-deficient cells (P388/CPT45) exhibit less staurosporine-induced DNA fragmentation (Fig. 5D) and internucleosomal DNA laddering (Fig. 5E) than the parental cells (P388). These results should be interpreted with caution because P388/CPT45 were selected by continuous exposure to camptothecin (31), and other molecular changes
within the apoptotic pathway may contribute to apoptotic defects in camptothecin-resistant cells (39).

In conclusion, our findings raise the possibility that Top1, which is abundant and ubiquitous in mammalian cells, could participate in apoptosis by directly generating DNA strand breaks. Like apoptotic endonucleases, Top1 is non-essential for apoptosis since Top1 silencing reduces but does not abrogate chromatin DNA fragmentation (Fig. 5C). However, the redundancy and the non-linearity of endonucleases activation during apoptosis might have evolved to confer a robust pathway ensuring that deficiency in one of the apoptotic nuclease is not sufficient to abrogate DNA fragmentation. Top1-DNA complexes could also engage the apoptotic machinery in trans as trapping of Top1 by camptothecins is among the most efficient inducers of apoptosis (40). Apoptotic Top1-DNA complexes could therefore serve to amplify the apoptotic process engaged by staurosporine (see Fig. 6), as well as other agents including arsenic trioxide (18) and UV irradiation (19).

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Figure legends

Figure 1. Staurosporine induces cellular Top1-DNA complexes. (A) Human leukemia CEM cells were treated for 5 h with 1 µM staurosporine (STP). Cesium chloride fractions collected from the bottom of the tube were subjected to Top1 immunoblotting. The DNA-containing fractions [7-10] are indicated by the bracket. (B) 3'2P-end-labeled DNA was incubated with recombinant human Top1 (rhTop1) in the absence (lane 2) or presence of camptothecin [CPT, 0.1 µM (lane 3) and 1 µM (lane 4)] or staurosporine [STP, 1 µM (lane 5) and 10 µM (lane 6)]. Lane 1, DNA alone. Top1-mediated DNA cleavage fragments are indicated by the arrowheads.

Figure 2. Staurosporine induces Top1-DNA complexes in cells undergoing apoptosis. Human leukemia CEM (A-C) and colon carcinoma HCT116 (D-F) cells were treated with 1 µM staurosporine for the indicated times. (A, D) Detection of Top1-DNA complexes in the DNA-containing fractions [7-10]. Camptothecin (CPT, 1 µM, 1 h) was used as a positive control in panel A. (B, E) Apoptotic DNA fragmentation was quantified by filter elution (mean ± S.D. of triplicate samples). (C, F) Top panel: DEVD-AFC peptide cleavage activity (mean ± S.D. of triplicate samples). Bottom panel: Western blot analysis of PARP in whole cell extracts. Numbers are molecular masses in kilodaltons. * indicates PARP cleavage product.

Figure 3. Staurosporine induces oxidative DNA lesions. (A) CEM cells were untreated (◻, ■) or treated with 1 µM staurosporine for 2 h (○, ●) and exposed (■, ●) or not (◻,
Staurosporine induces apoptotic Top1-DNA complexes, p. 21 of 22

(Ø) to Fpg for 30 min. DNA single-strand breaks were assayed by alkaline elution. (B) Detection of AP sites in isolated nuclei from staurosporine-treated (1 µM, 1 h) HL-60 cells incubated in the absence (lane 1) or presence of recombinant Ape1 (lane 3) for 90 min. DNA breaks were detected by agarose gel electrophoresis. Recombinant Ape1 was also incubated for 90 min with isolated nuclei from untreated HL-60 cells (lane 2). (C) A 3'-32P-end-labeled 23-bp oligonucleotide with a canonical Top1 cleavage site was incubated with recombinant human Top1 (rhTop1) or nuclear extracts from untreated (control) or staurosporine-treated (STP, 0.1 µM, 3 h) CEM cells in the absence (-) or presence (+) of 10 µM camptothecin. DNA, oligonucleotide alone. Top1-mediated cleavage generates a 13 nucleotides fragment indicated by the arrowhead.

Figure 4. The antioxidant N-acetyl-L-cysteine and the caspase inhibitor Z-VAD-fmk prevent staurosporine-induced Top1-DNA complexes. CEM cells were untreated (control) or preincubated with N-acetyl-L-cysteine (NAC, 30 mM, 1 h) or Z-VAD-fmk (100 µM, 30 min) before the addition of staurosporine (STP, 1 µM, 6 h). (A) Cells were visualized by phase contrast microscopy (a-d). ROS were visualized by DCF fluorescence of the same fields (e-h). (B) Quantification of DCF fluorescence per cell (mean ± S.D. of 100 individual cells). (C) Detection of Top1-DNA complexes in the DNA-containing fractions [6-9].

Figure 5. Involvement of Top1 in DNA fragmentation during staurosporine-induced apoptosis. (A) CEM cells were preincubated with or without N-acetyl-L-cysteine (NAC, 30 mM, 1 h) before the addition of staurosporine (STP, 1 µM) for the indicated times.
Apoptotic DNA fragmentation was quantified by filter elution (mean ± S.D. of triplicate samples). (B,C) Top1 was silenced in HCT116 cells using U6 promoter-driven DNA vectors stably expressing siRNA hairpins targeting human Top1 (siRNA-Top1) or a negative control sequence (siRNA-control). (B) Western blot analysis of Top1 and actin in whole cell extracts. Numbers are molecular masses in kilodaltons. (C) HCT116 cells were treated with 0.1 μM staurosporine (STP) for the indicated times and DNA content was analyzed by flow cytometry. Sub-G1 cells are indicated by the brackets. Numbers correspond to percentages of cells with sub-G1 DNA. (D) Parental (P388, ●) and Top1-deficient (P388/CPT45, ○) cells were treated with 0.1 μM staurosporine for the indicated times. Apoptotic DNA fragmentation was quantified by filter elution (mean ± S.D. of triplicate samples). (E) Visualization of apoptotic DNA fragmentation by agarose gel electrophoresis in P388 (lane 1 and 2) and P388/CPT45 cells (lane 3 and 4) treated (+) or not (-) with 1 μM staurosporine (STP) for 4 h. Lane 5, marker. Numbers indicate the size of marker DNA fragments in base pair.

**Figure 6. Proposed mechanism for the induction of Top1-DNA complexes during staurosporine-induced apoptosis.** Staurosporine induces the caspase-dependent generation of reactive oxygen species (ROS) that produce DNA lesions, which in turn generate Top1-DNA complexes. Top1-DNA complexes contribute to chromatin degradation by generating DNA strand breaks and by activating caspases.
Staurosporine induces apoptotic Top1-DNA complexes

Figure 1
Figure 2
Figure 3
Figure 4

Staurosporine induces apoptotic Top1-DNA complexes
Figure 5

Staurosporine induces apoptotic Top1-DNA complexes
Staurosporine induces apoptotic Top1-DNA complexes

Figure 6
Supplementary figure 2. Staurosporine (STP) induces Top1-DNA complexes in HL-60 cells undergoing apoptosis (1 µM, 3 h) (ref. 32). Detection of Top1-DNA complexes in the DNA-containing fractions [fractions 7-10].
**Supplementary figure 5.** Top1 silencing reduces staurosporine-induced DNA fragmentation. Top1 was silenced in HCT116 cells using U6 promoter-driven DNA vectors stably expressing siRNA hairpins targeting human Top1 (siRNA-Top1) (cDNA sequence: 5’-AAC CTC CAA ACT CAA TTA T-3’) or a negative control sequence (siRNA-control) (cDNA sequence: 5’-GCG TCC TTT CCA GAT A-3’). (A) Western blot analysis of Top1 in whole cell extracts. Number is molecular mass in kilodaltons. (B) HCT116 cells were treated with 0.1 µM staurosporine for the indicated times and DNA content was analyzed by flow cytometry. Sub-G₁ cells are indicated by the brackets. Numbers = percentage of cells with sub-G₁ DNA.
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