Cloning of Chinese Hamster DNA Topoisomerase I cDNA and Identification of a Single Point Mutation Responsible for Camptothecin Resistance*

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A camptothecin-resistant (DC3F/C-10) Chinese hamster cell line that contains a catalytically altered and camptothecin (CPT)-resistant DNA topoisomerase I (top 1) (Tanizawa, A., and Pommier, Y. (1992) Cancer Res. 52, 1848–1854) and the parent cell line (DC3F) were used to compare top 1 mRNAs and cDNAs. Northern blot analysis showed a single 4.1-kilobase band without quantitative reduction between the two cell lines. We have cloned and sequenced top 1 cDNAs. DC3F and DC3F/C-10 top 1 c-DNA are 3591 and 3626 base pair long, respectively, and encode 767 amino acids. The homology of deduced amino acid sequences between Chinese hamster and mouse or human top 1 are 98.1 and 96.7, respectively. cDNAs from DC3F/C-10 and DC3F cells differ by a single base point mutation (G to A) which results in an amino acid change from Gly505 to Ser (Gly505 → Ser). Gly505 corresponds to Gly506 of human top 1 cDNA and is located 220 amino acids away from the presumed catalytic Tyr726. The point mutation in the Chinese hamster top 1 is located in a region that is highly conserved among all cloned top 1 cDNAs (plant ATH, vaccinia virus, Shope fibroma virus, Drosophila, Saccharomyces cerevisiae, Schizosaccharomyces pombe, mouse, and Human). A mutation of Asp563 to Gly in this same region has been shown to confer CPT resistance for human top 1. Chinese hamster top 1 protein with a Gly563 → Ser mutation that was expressed in bacteria was resistant to CPT, indicating that this single base mutation is involved in CPT resistance. Our results suggest that the highly conserved region around Gly563 plays an important role in the interactions among top 1, DNA, and CPT.

Camptothecin (CPT)1 is a DNA topoisomerase I (top 1) inhibitor (1–4), and its derivatives are among the most promising anti-cancer agents currently in clinical trials (5). Despite the experimental evidence that CPT binds to top 1-DNA complexes rather than to purified DNA or top 1 enzyme (6), the molecular interactions between the drug and its presumed receptor site remain hypothetical (4). DNA interaction is suggested by CPT’s strong preference for guanine at the 5’ terminus of the stabilized top 1-induced DNA breaks (7), camptothecin-induced DNA photodamage experiments (8), and protein interaction by photolabeling experiments (9).

Based on the findings obtained with several CPT-resistant cell lines, a decrease in the amount of top 1 protein seems to be the main mechanism for drug resistance (10). However, several other CPT-resistant cell lines exhibit either chromosomal rearrangement (11–14) or top 1 point mutation (15–16). Formation of top 1-mediated cleavable complex and replication fork arrest have been reported as the initial steps in the cascade leading to drug-induced cell death (17–19).

In an attempt to characterize the top 1 regions involved in CPT activity and to sequence for the first time the Chinese hamster top 1 enzyme, we have isolated the top 1 cDNAs from wild type (DC3F) and CPT-resistant (DC3F/C-10) Chinese hamster lung fibroblasts (20). We have reported previously that the amount of top 1 enzyme from DC3F/C-10 cells is increased rather than reduced and that its molecular size is similar (approximately 100 and 68 kDa) when compared with top 1 from parental DC3F cells. Furthermore, top 1 from DC3F/C-10 cells was CPT-resistant and showed reduced catalytic activity with approximately 5-fold less specific activity than that from parental cells (20).

MATERIALS AND METHODS

Cell Culture and Bacteria—The Chinese hamster lung fibroblast DC3F cell line and its CPT-resistant subline (DC3F/C-10) were grown in minimum essential medium with Earle’s salt, supplemented with 10% of heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (ABI, Columbia, MD) as described previously (20).

Escherichia coli XL1-Blue strain (recA1, lac– endA1, gyrA96, thi, hsdR17, supE44, relA1, F' proAB, lacI, lacZ ΔM15, Tn10) was used for cloning and sequencing top 1 cDNA. Plasmids for protein expression were transfected to TOP10F' strain (mcrA, ΔimrR had Rms– lacBCl86lacZΔM15, ΔexZ74, deoR, recA1, araD139, Δara, leu2, 7697,galU, galK, x,ompT, endA1, nupG, F').

Construction and Screening of cDNA Libraries—Total cellular RNAs of both DC3F and DC3F/C-10 cells were extracted using the

1 The abbreviations used are: CPT, camptothecin; top 1, DNA topoisomerase I; kb, kilobase(s); IPTG, isopropyl-1-thio-β-D-galactopyranoside; bp, base pair(s); PCR, polymerase chain reaction.
guanidinium thiocyanate-phenol-chloroform extraction method (21). Poly(A) RNA was purified with oligo(dT)-cellulose columns. The first cDNA libraries were constructed with the ZAP-cDNA synthesis kit using oligo(dT) primer (Stratagene, La Jolla, CA) which was kindly provided by Dr. Earnshaw (Johns Hopkins University) (22). Since the longest clone was approximately 2.5 kilobase (kb) in the case of both DC3F clone WTSF8 and DC3F/C-10 (clone TAF4) cells (see Fig. 2), we constructed a second set of cDNA libraries using a specific primer for DC3F top 1 cDNA (5'-TTTCCGATGTTGTCC AC-3'), from position 1465 to 1449 (see Fig. 3) to obtain the 5'-end of cDNA. The second set of cDNA libraries from DC3F and DC3F/C-10 cells was screened with the EcoRI/NdeI fragment of TIB (0.9 kb, from position 2 to 901). The longest clones from DC3F and DC3F/C-10 cells were designated as WTS12 and TAS17, respectively. Although TAS17 included the open reading frame, WTSl2 did not and a specific primer for DC3F top 1 cDNA (289 bp) was obtained and sequenced using PCR technique as described under “Results” (WTP2).

**Blot Hybridization and Sequence Determination—**Blot hybridization was performed according to standard procedures. Blots were hybridized with Chinese hamster top 1 cDNA (1.7-kb fragment of WFT8, nucleotide 1061–2801) or with a β-actin oligonucleotide probe (Oncogene Science, Inc., Manhasset, NY), which had been labeled with [32P]dCTP (Du Pont NEN) by random priming procedure, at 42 °C in the presence of 50% formamide and washed at the same temperature with a solution of 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 0.1% sodium dodecyl sulfate (SDS), and 0.25 x SSC, 0.1% SDS. Signal intensities were compared using a Betascope 603 blot analyzer (Betagen Inc.). The cDNA sequences were determined by the dideoxynucleotide chain-termination method using Taq DNA polymerase (Stratagene, La Jolla, CA) and either 32P- or 33P[dATP (DuPont NEN)].

**Construction of Protein Expression Plasmids for Top 1—**A recombinant plasmid containing the full-length top 1 cDNA from DC3F/C-10 cells was first constructed using TAS17 and TAF4. Thereafter, a truncated top 1 cDNA containing the entire amino acids coding region (position 107 to the end) was inserted into pTrcHis A (PE/CI) (position from 1256 to 2499), which contained the mutation site, was replaced with the counterpart of Bsu36I fragment of PE/CI. As for the expression of top 1 from DC3F/C-10 cells, a truncated top 1 cDNA containing the entire amino acids coding region (position 107 to the end) was inserted into pTrcHis A (PE/CI) and either EcoRI or PstI (Promega) was digested with the indicated enzymes (WTP2).

**Expression of Recombinant Topoisomerase I—**Expressionally growing bacteria (OD600 0.7) were treated with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Stratagene, La Jolla, CA) for 1 h to express top 1. The bacteria in 50 ml of culture medium were spun down, resuspended in 5 ml of lysis solution (20 mM phosphate, 500 mM NaCl, pH 7.8), and treated with egg white lysozyme (100 μg/ml) (Boehringer Mannheim) for 15 min on ice. The cells were disrupted by three cycles of sonication and freeze-thawing. The cell lysate was treated with 5 μg/ml RNase (Sigma) for 15 min on ice. After centrifugation at 3000 x g for 15 min, the supernatant was used for catalytic assay. Measurement of Topoisomerase I Activity—Catalytic activity was examined in the absence of Mg2+ to avoid influence of bacterial enzymes (26). SV40 DNA (0.4 μg) (Life Technologies, Inc.) was used as a control. The labeled plasmid was incubated with the cell lysate in 10 μl of reaction mixture (30 mM Tris, 150 mM KCl, 1 mM EDTA, pH 7.5) for 5 min at room temperature, reactions were stopped by adding 3 μl of 10 x loading buffer (final concentrations: SDS, 0.1%; EDTA, 10 mM; Ficoll, 2%; bromphenol blue, 0.025%). DNA was analyzed by 1% agarose gel electrophoresis in Tris/phosphate/EDTA buffer containing 2 μg/ml chloroquine as described previously (27).

**DNA Oligonucleotide Cleavage Assays—**The following double-stranded oligonucleotide was used.

5'-GATCTAAGAACTTG GTAGAAAATTTTTTTTTTTTACAG-3'

**SEQUENCE 1**

This oligonucleotide is derived from the Tetrahymena ribosomal DNA sequence containing a strong top 1 cleavage site (A) (24), in which the +1 base has been mutated to a G (italic letters) to enhance CPT cleavage (4, 7). 3'-End labeling of the upper strand was performed by using terminal deoxynucleotidyl transferase and 32P-labeled cordycepin (³²P) (Du Pont NEN) as described previously (7). Reactions were performed in 10 μl of reaction buffer (0.01 M Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 15 μg/ml bovine serum albumin) for 30 min at 20 °C and were stopped by adding sodium dodecyl sulfate (0.5% final concentration) and 40 μl of loading buffer (80% formamide, 19 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue). Four microliters were loaded into 16% acrylamide/um sequencing gels. At the end of electrophoresis, gels were transferred to Whatman 3MM paper sheets, dried, and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The full-length end-labeled oligonucleotide (33-mer, including cordycepin) was converted to a 19-mer after camptothecin-induced cleavage (caret between T and G on the upper strand shown above).

**RESULTS**

**Northern Blot Analysis of Total RNA for Top 1 Message—**Total cellular RNAs from DC3F and DC3F/C-10 cells which had been transferred onto nitrocellulose filters were examined as described under "Materials and Methods" (Fig. 1A). The same blot was re-hybridized with a 40-base human β-actin oligonucleotide probe as an internal control. As shown in Fig. 1A, the top 1 probe detected a 4.1-kb signal, and there was no size difference between top 1 messages from DC3F and DC3F/C-10 cells. Comparison of signal intensities revealed that ratios of top 1 to actin message in DC3F/C-10 RNA were approximately 1.8-fold greater than those in DC3F cells.

**Southern Blot Analysis for Genomic Top 1 DNA—**Genomic DNA was extracted from DC3F and DC3F/C-10 cells using the salt extraction method (25). DNA (10 μg) was digested with the indicated enzymes (Fig. 1B), separated on 0.7% agarose gels, and transferred to nitrocellulose membranes. The membranes were hybridized with Chinese hamster partial top 1 cDNA. No difference was detected between restriction maps of the top 1 genes from DC3F and DC3F/C-10 cells. This suggests that there is no chromosomal rearrangement of
the top 1 gene in the DC3F/C-10 cell.

Isolation and Sequencing of the Top 1 cDNAs from Chinese Hamster Cells—As shown in Fig. 2, we first isolated partial top 1 cDNA clones from a first set of libraries prepared from DC3F and DC3F/C-10 cells. The clones were approximately 2.5 kb in length starting from the poly(A) tail. We reconstructed and screened the second set of libraries as described under “Materials and Methods.” An additional 1477-bp clone, which contains an open reading frame (TAS17), and a 1268-bp clone (WTS12) were obtained from the second set of libraries from DC3F/C-10 and DC3F cells, respectively. TAS17 and WTS12 lacked 18 and 50 bases at their 3’-ends which contain complementary sequences to the primer. To obtain base sequences of the missing amino terminus of top 1 cDNA from DC3F cells, a 15-mer oligonucleotide (5’-TGCTCTCGGGAACT-3’) which corresponds to base sequences of TAS17 (from position 16 to 30) was used as upstream primer for PCR reaction. After removal of external primers and unincorporated dNTPs, PCR products were directly sequenced with several internal primers (WTP2 segment in Fig. 2).

Recombinant plasmids containing the full-length top 1 cDNA from DC3F (PE/W1) and DC3F/C-10 cells (PE/C1) were constructed into pTrcHis A (PE/C1) (Invitrogen), as described under “Materials and Methods.”

Topoisomerase I cDNA of Chinese Hamster Cells—The entire top 1 cDNAs from DC3F and DC3F/C-10 cells were sequenced. Fig. 3 shows the nucleotide and deduced amino acid sequences of the top 1 cDNA from DC3F cells. Top 1 cDNA from DC3F cells is 3591 bp long and encodes 767 amino acids. The Chinese hamster cDNA contains 45 additional bases from the EcoRI recognition sequence, which corresponds to the 3’-end of human cDNA. As occurs in the mouse, Chinese hamster top 1 has 2 additional amino acids not present in the human. The nucleotide and deduced amino acid homologies between Chinese hamster and human are 86% and 97%, respectively.

**Comparison of the cDNA coding sequences from DC3F and DC3F/C-10 cells revealed a single base point mutation (G to A) which results in an amino acid change from Gly^505 to Ser^506 (Gly to Ser) (Fig. 4). Gly^505 corresponds to Gly^500 coded for in human top 1 cDNA (Figs. 2 and 7). Also, Top 1 cDNA from DC3F/C-10 cells has 30 additional bases at the 5’-end (5’-TGCTGCCC-GTTCGTCTGGCTCTCGGGAACT-3’) and 5 additional bases at the 3’-end (5’-AACGG-3’) when compared with that from DC3F cells. However, these changes did not affect the amino acid sequence. Taken together, our results show that CPT resistance in DC3F/C-10 cells is associated with a single amino acid change, Gly^506 to Ser.

**Catalytic Activity of Expressed Top 1 in Bacteria—**Once IPTG was added to culture medium, OD_600 values showed a transient increase for 1 h, but thereafter decreased down to the initial value (data not shown). As negative control, pTrcHisA with top 1 cDNA from DC3F/C-10 cells in the reverse orientation was transfected to TOP10F’ and analyzed in parallel (PE/C1rev). As reported previously (23), expression of top 1 protein was toxic to host bacteria.

Lysates were prepared from 50 ml of culture of IPTG-treated bacteria carrying PE/W1, PE/C1, or PE/C1rev. As shown in Fig. 5, CPT inhibited the relaxation of supercoiled SV40 DNA by the cell lysate from E. coli harboring the PE/W1 plasmid. Significant inhibition was detectable at 5 µM CPT concentration and above. By contrast, PE/C1 top 1 catalytic activity was resistant to CPT up to 100 µM (Fig. 5, lower panel). In addition, the catalytic activity from the PE/C1 plasmid was weaker than that from the wild type enzyme (3–5-fold). The lack of magnesium-independent DNA religating activity of the cell lysate from E. coli transformed by the PE/C1rev plasmid indicated that the observed catalytic activity in the cell lysates from PE/W1 and PE/C1 cultures was due to expressed Chinese hamster top 1 proteins (data not shown). Western blot analysis of the cell lysates was carried out using human scleroderma serum which was kindly provided by Dr. Earnshaw (Johns Hopkins University). When compared with cell lysate from bacteria carrying PE/C1rev, several additional bands were detected. The largest one was approximately 104 kDa, which is consistent with the expected size of the fusion protein. There was no difference between the size of immunoreactive proteins in the cell lysates from bacteria carrying CPT-sensitive and -resistant top 1 (data not shown).

**Comparison of the cDNA coding sequences from DC3F and DC3F/C-10 cells revealed a single base point mutation (G to A) which results in an amino acid change from Gly^505 to Ser^506 (Gly to Ser) (Fig. 4). Gly^505 corresponds to Gly^500 coded for in human top 1 cDNA (Figs. 2 and 7). Also, Top 1 cDNA from DC3F/C-10 cells has 30 additional bases at the 5’-end (5’-TGCTGCCC-GTTCGTCTGGCTCTCGGGAACT-3’) and 5 additional bases at the 3’-end (5’-AACGG-3’) when compared with that from DC3F cells. However, these changes did not affect the amino acid sequence. Taken together, our results show that CPT resistance in DC3F/C-10 cells is associated with a single amino acid change, Gly^506 to Ser.

DISCUSSION

In this study, we cloned the entire top 1 cDNAs from CPT-resistant (DC3F/C-10) and its parental Chinese hamster lung fibroblast cells (DC3F) and found a single amino acid substitution in top 1 cDNA from DC3F/C-10 cells (Gly^506 to Ser). Relaxation of DNA by cell lysates containing expressed recombinant proteins in the presence of CPT suggests that this
FIG. 3. Nucleotide and deduced amino acid sequences of Chinese hamster DC3F top 1 cDNA. Chinese hamster DC3F top 1 cDNA encodes 767 amino acids. Position of catalytic Tyr is deduced by alignment and underlined. The single amino acid mutation (Gly to Ser) in DC3F/C-10 top 1 cDNA is shown in a box.

5' 3'
G-C
4
Gly
3' 5'
C-T
G-A
T-A
G

DC3F
DC3F/C-10

ACGT
ACGT

G-C
G

T-A
A

Gly
Ser

Top 1 cDNA and CPT Resistance

FIG. 4. Single point mutation in top 1 cDNA from DC3F/C-10 cells. Base sequences were determined by dideoxynucleotide chain termination method. At least two independent clones were used for sequence determination. A representative sequencing ladder illustrating the single base mutation (G to A) is shown. This mutation results in an amino acid change from Gly to Ser and corresponds to Gly505 of human top 1 cDNA.

mutation is responsible for CPT resistance.

As shown in Fig. 2, comparison of human and Chinese hamster top 1 demonstrated 24 amino acid differences, including 2 additional amino acids in Chinese hamster top 1. Nineteen of the 24 differences are coded for between positions 41 and 349 of the Chinese hamster amino acid sequence. Moreover, comparison of mouse top 1 amino acid sequence with that for wild type Chinese hamster DC3F reveals 14 amino acid substitutions that are all located between positions 177 and 313. These findings indicate a greater conservation in C-G Ser of the carboxyl terminus than in the amino terminus.
by continuous exposure to CPT-11 (26). The deduced amino acid sequence for CPT-K5 top 1 cDNA indicated two substitutions (Asp$^{533}$ to Gly and Asp$^{533}$ to Gly) (26). However, site-directed mutagenesis analysis revealed that only Asp$^{533}$ → Gly is responsible for CPT resistance (27). The other CPT-resistant cell line is the non-small cell lung cancer cell line PC-7/CPT which shows a single amino acid change of Thr$^{279}$ to Ala (28). The three mutations found to date can be viewed in terms of their location. While Thr$^{279}$ → Ala is very close to catalytic Tyr$^{272}$, Asp$^{533}$ → Gly and Gly$^{505}$ → Ser (which corresponds to Gly$^{505}$ in human top 1 (Fig. 7) are relatively far from the catalytic tyrosine but close to each other. As shown in Fig. 7, both of the mutations Asp$^{533}$ → Gly and Gly$^{505}$ → Ser are located in a highly conserved region among all the sequenced top 1 genes (human (22), mouse (29), Chinese hamster (this paper), vaccinia virus (30), Shope fibroma virus (31), S. cerevisiae (32), S. pombe (33), Drosophila (34), plant (Arabidopsis thaliana) (GenBank accession number X57544). This similarity suggests that this highly conserved region probably plays an important role for the interaction of top 1 with its DNA substrate.

Morham and Shuman (35) reported extensive work on seven mutants of vaccinia top 1. Four mutants had changes located within the conserved region shown in Fig. 7 (Gly$^{392}$ → Ser, Gly$^{392}$ → Asp, Thr$^{442}$ → Ile, and Thr$^{447}$ → Ile). Mutant proteins with Ser$^{392}$ or Ile$^{447}$ exhibited substantial reduction of DNA relaxation activity. The Asp$^{392}$ mutant was inert with respect to DNA relaxation, whereas the Ile$^{447}$ mutation had less effect on activity than the other mutations. These four mutants are within the conserved region involved in CPT resistance for DC3F/C-10 and CPT-K5 top 1. The reduced specific activity of mutant top 1 from DC3F/C-10 cells is consistent with the mutant vaccinia top 1 data, indicating that the conserved noncatalytic region is critical for catalytic function. These results suggest that the top 1 catalytic site may consist of several protein domains, including the catalytic tyrosine region and the conserved region shown in Fig. 7. Hence, CPT resistance of mutant top 1 from DC3F/C-10 cells could be due to an altered secondary structure and protein conformation change which could affect the interaction of top 1 with the top 1-DNA complex.

In the case of the human top 1 mutant from CPT-K5 cells, the authors speculated that the specific activity of mutant top 1 is similar to that of wild type top 1, based on the results of Western blot analysis and total top 1 activity in the crude extracts (27). It is possible that amino acid substitution from Asp$^{533}$ to Gly in human top 1 has a different effect than the other noncatalytic mutations listed in Fig. 7, even though they are relatively close each other. The predicted conformational change of mutant top 1 in CPT-K5 suggested that the mutated site becomes recessed from the surface in a protruding region of the molecule (28). We analyzed the possible conformational alteration of mutant top 1 due to the Gly$^{505}$ → Ser mutation, using the Gene Works software program (IntelliGenetics, Mountain View, CA) and found some differences between CPT-sensitive and -resistant top 1 in terms of Garnier protein structure predictions α and β (36). However, it is not clear whether these differences are relevant to CPT resistance and reduced specific activity. It is possible that the
glicine to serine substitution reduces the flexibility around the wild type Gly265 residue.

Amino acids surrounding the catalytic tyrosine compose another region where mutations can alter specific activity and CPT sensitivity, for example amino acid substitution from Thr270 to Ala in PC-7/CPT cells (15). Replacement of the vaccinia virus top 1 active tyrosine site region (SKRAY) with the counterpart of yeast top 1 (SKNY) does not make vaccinia top 1 sensitive to CPT, whereas the mutated yeast top 1, which is conserved among all the known top 1. The counterpart of yeast top 1 (SKINY) does not make vaccinia sensitive to CPT, because the Asp321 sensitivity where there is no homology between yeast and vaccinia top 1 sensitive to CPT, whereas the mutated yeast top 1, which is conserved among all the known top 1.

Another possible approach to analyzing top 1 mutant is to compare the effects of CPT derivatives on mutant 1. Structure-activity studies of CPT derivatives are consistent with the existence of a stereospecific drug binding pocket (3, 4, 38, 39).

CPT-resistant top 1 might still be sensitive to other CPT analogues. Such an analysis may provide useful information on the molecular mechanism of drug-induced inhibition of top 1 activity and on ways to overcome such resistance in cancer chemotherapy.

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