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Research Paper

Directed evolution to increase camptothecin sensitivity of human DNA topoisomerase I

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Abstract

Background: Human DNA topoisomerase I (top1) relaxes DNA supercoiling during basic nuclear processes. The enzyme is the main target of antitumor agents, such as camptothecins (CPT), that transform top1 into a DNA-damaging agent.

Results: By directed evolution of a C-terminal portion, we selected human top1 mutants that were 22–28-fold more CPT-sensitive than wild-type top1 in *Saccharomyces cerevisiae* cells. The evolved enzymes showed unique mutation patterns and were more processive in plasmid relaxation assays. A top1 mutant had only two amino acid changes in the linker domain, one of which may change a linker/core domain contact surface. The mutant stimulated DNA cleavage to higher levels than the wild-type enzyme and was more sensitive to CPT in a cleavage assay. Moreover, the mutant was more CPT-sensitive than wild-type top1 in a repair-deficient yeast strain.

Conclusions: Mutations in the linker domain can affect DNA binding and CPT sensitivity of human top1. Such drug-hypersensitive topoisomerases may be useful in developing DNA cutters with high cell lethality and in new drug discovery programs.

Keywords: Camptothecin; DNA shuffling; Drug action; Enzyme mutant; Topoisomerase I-B

1. Introduction

Engineered DNA-binding proteins with novel characteristics, such as site specificity, ligand-dependent effects, specific protein interactions and others, can have broad applications in the post-genomic era. Combinatorial approaches have been used to select transcription factors with a pre-defined site selectivity, thus allowing the targeting and selective regulation of a few genes of interest [1]. Directed evolution by DNA shuffling is most powerful in selecting mutant proteins with novel functions, new biosynthetic pathways [2–5] and viral genomes with increased cell-specific infectivity [6]. The basic technique explores the sequence space of possible mutants in a combinatorial manner, therefore avoiding a rational approach to develop new protein functions.

Attempts to modify DNA-binding proteins other than transcription factors have been limited. Recently, we have shown that it is possible to alter the site selectivity of human DNA topoisomerase I (top1) by fusion to the DNA-binding domain of the yeast Gal4 transcription factor [7]. Top1 is an essential enzyme in multi-cellular organisms, though it is dispensable in yeast [8–10]. It relaxes supercoiled DNA during several important nuclear processes, such as replication and transcription, by coupling DNA breaking and rejoining activities [8,9]. Top1 introduces single-stranded breaks in a duplex, and before resealing the cut, it allows the free end of the broken strand to rotate around the intact strand resulting in relaxation of superhelical tension [11,12]. Human top1 is constituted by several domains (Fig. 1A), and crystal structures showed that it clamps fully around a double helix with the non-conserved linker domain protruding from one side of the clamp [11,13]. The linker domain is constituted by two α-helices and may contact DNA modulating the rotation of the broken strand around the other [11–13].

Top1 is also the cellular target of the antitumor drug

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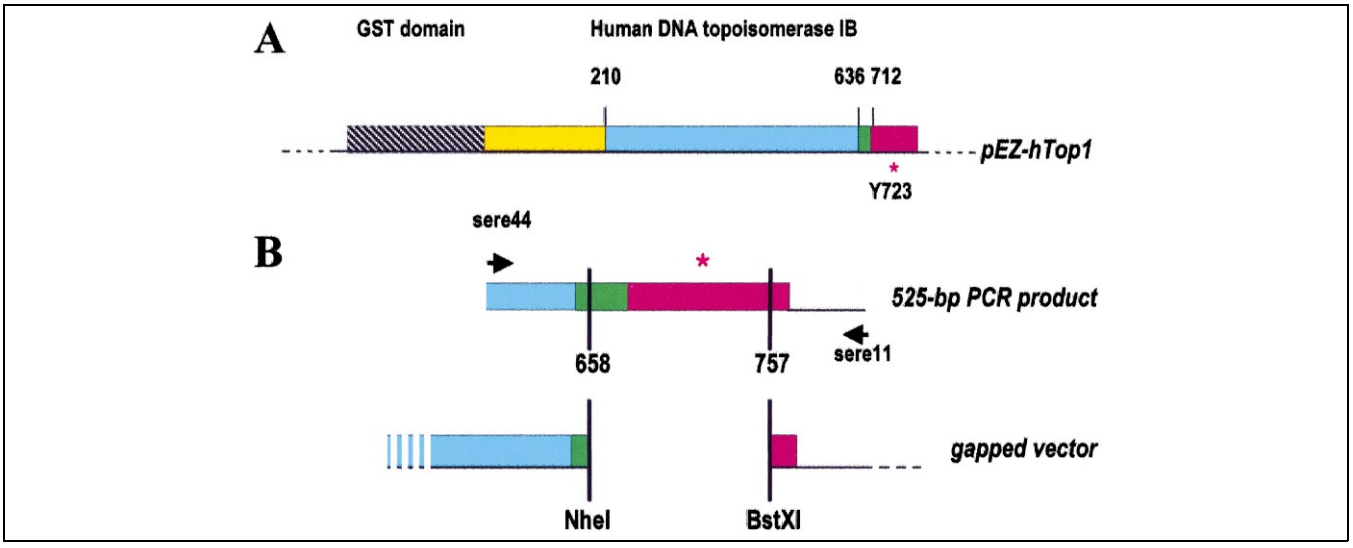

Fig. 1. Human top1 domains and evolution strategy. (A) A portion of the 2-μ-based pEZ-hTop1 plasmid is shown indicating the expressed GST-fused human top1. Protein domains are marked by colors: yellow, the N-terminal domain fused to the GSH-binding domain of GST (dashed box); blue, the DNA-binding core domain; green, the non-conserved linker domain; and red, the C-terminal domain including the active 723-tyrosine residue (asterisk). Numbers indicate the amino acids at domain boundaries. (B) An error-prone PCR was performed by using primers sere44 and sere11 that amplify a fragment of 525 bp, including the region from amino acids 658 to 757. PCR products were transformed into yeast cells together with a gapped vector, prepared by digesting the pEZ-hTop1 plasmid with *Nhe*I and *Bst*XI endonucleases that have unique restriction sites as indicated.

camptothecin (CPT) that hinders the rejoining reaction, thus stabilizing a transient DNA–protein complex, wherein a DNA strand is cut and covalently linked to a tyrosine residue [9,14,15]. Trapped DNA–top1 complexes can eventually collide with advancing replication forks generating irreversible double-stranded DNA cuts and cell death [9,16]. Therefore, drug action transforms a 'good' enzyme into a 'bad' protein that damages the DNA [14,15,17,18]. Our long-term goal is to exploit the inherent potential of top1 to inflict a great harm to a cellular genome to develop a nuclear toxin that can efficiently kill cancer cells [7]. Here, we have explored the possibility of developing a top1 enzyme that is normally functional, yet more prone to be transformed into a DNA-damaging agent by CPT. Thus, we applied DNA shuffling methodology to increase CPT sensitivity of human top1 expressed in yeast.

2. Results

2.1. Library preparation

Yeast cells lacking the TOP1 gene are viable and completely resistant to CPT. Consistently, the re-introduction of a wild-type (wt) top1 restored CPT sensitivity of cells [20,24] (see also Fig. 2). Therefore, we have used a *Saccharomyces cerevisiae* strain, JEL1-Δ*top1*, that lacks a functional top1 [19] to select top1 mutants. The 2-μ-based pEZ-hTop1 plasmid carries a human wt top1 cDNA that is expressed at low levels when JEL1-Δ*top1* cells are grown in glucose, due to the leakyness of the promoter [25]. That confers a slight CPT sensitivity to cells (Fig. 2), thus allowing selection for enhanced sensitivity. In this work, we evolved a protein segment of 100 amino acids that included the linker and C-terminal domains (Fig. 1B) by a DNA shuffling technique [2–5]. Evolution cycles were repeated three times, and at the second and third cycles, the selected clones of the first and second cycles, respectively, were used as parent clones. A 525-bp fragment was amplified by error-prone PCR from parent clones, digested with DNase I and shuffled by primer-less PCR. Then, the full-length DNA was amplified and cloned into a gapped

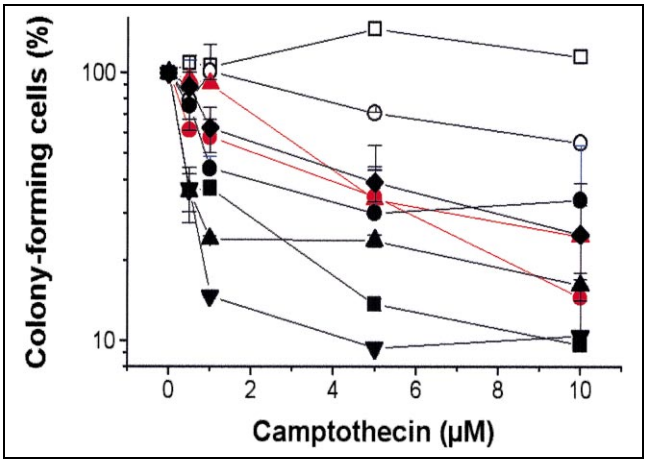

Fig. 2. Enhanced drug sensitivity phenotype of evolved top1s in yeast cells. Cytotoxicity of CPT in cells expressing top1 mutants was determined with a clonogenic assay. Colony-forming cells are reported as percentages of control cells. Symbols are: open square, no top1; open circle, wt top1; red circle, 5-II; red triangle, 89-II; black circle, 15-III; black diamond, 51-III; black up-triangle, 43-III; black down-triangle, 50-III; and black square, 50-III. Bars show standard error of the mean.

Table 1
CPT sensitivity of yeast cells expressing the selected enzymes and amino acid mutations of evolved top1s

Top1	CPT cytotoxicity[a]		Amino acid mutations[c]
	ID_{50} (μM)	Increase (-fold)[b]	
wt	>10	–	
26-I	8	1.3	K679R
45-I	8.7	1.2	R663G, D677G
32-I	9.4	1.1	A661G
25-I	10	1.0	V683I
5-II	1	10	D677G, V703I
89-II	3.4	2.9	R663G, K669R, V683I, K712R, I728T
43-III	0.35	28	R663G, K669R, **D677G**, **V703I**, **I728T**, T747A
53-III	0.40	25	R663G, K669R, **D677G**, **V703I**
50-III	0.45	22	**D677G**, **V703I**, Q704L, **I728T**
15-III	0.9	11	**D677G**, **V703I**, K712R, **I728T**
26-III	1.8	5.5	**V703I**, **I728T**, K742R
51-III	3.2	3.1	R663G, **D677G**, **V703I**, **I728T**

[a]CPT cytotoxicity was measured in JEL1-Δ*top1* cells bearing evolved top1s with a clonogenic test. ID_{50} values are drug concentrations inhibiting colony formation of treated cells to 50% of control cells. They were determined from the curves shown in Fig. 2. Drug sensitivity of clones selected at the first cycles were only slightly different from wt top1.
[b]Fold increase over wt top1. Since the wt value is higher than 10 μM, ratio values are also higher than the listed ones.
[c]For the selected clones of the third cycle (III), amino acid mutations in bold were detected at higher than expected frequency, which corresponded to 0.5 since the two selected clones of the second cycle (II) were used as parents in the third cycle. The underlined mutations were at lower than expected frequency in the same clone set.

vector by homologous recombination in JEL1-Δ*top1* cells (Fig. 1B). A library of about 10^4 clones was obtained at each cycle, and clones were selected with a multiple-step procedure utilizing gradually more sensitive cytotoxicity assays (see Section 4 for details). At the first, second and third cycles, we selected four, two and six mutants, respectively (Table 1).

2.2. CPT sensitivity of selected top1 mutants

After the clone selection process, the corresponding vectors were isolated and further studied. First, CPT sensitivity of JEL1-Δ*top1* cells expressing the selected top1s was determined with a clonogenic assay. The four top1 clones of the first cycle were only slightly more sensitive to CPT than the wt enzyme (Table 1). Nevertheless, top1s from the second and third cycles conferred a marked higher CPT sensitivity to yeast cells than wt top1, particularly at low drug concentrations (Fig. 2 and Table 1). CPT potency was different depending on the expressed enzyme: 5-II, 43-III, 53-III and 50-III enzymes sensitized yeast from 10- to 28-fold more than wt top1. Interestingly, the rate of increased drug sensitivity was clearly diverse at the three cycles: we detected the greatest increase (10-fold for 5-II) at the second cycle, whereas little increase was achieved at the first cycle, and the best mutant (43-III, obtained at the third cycle) improved only three-fold relative to 5-II (Table 1). It can also be noted that 51-III, 26-III, and 15-III showed a drug sensitivity similar to their parent clones (5-II and 89-II). To test whether mutations in other regions of the vector affected cytotoxicity results, the evolved region was swapped between 5-II, 89-II, 43-III and 50-III, on one side, and wt top1, on the other. Swapped vectors conferred an enhanced CPT sensitivity to cells (not shown) to the same extent of the corresponding evolved clones, thus suggesting that amino acid sequences of the shuffled protein portion (Fig. 1B) were likely responsible for the new phenotype.

2.3. Amino acid mutations of evolved top1s

The evolved regions of the 12 selected top1 cDNAs were fully sequenced. In addition, we determined base sequences of five clones that were selected up to the last step of selection procedures, and were identical to wt top1 in cytotoxicity assays (not shown). In these clones we found 28 base mutations (25 transitions and three transversions) and no base deletions or insertions. We also sequenced eight random, unselected clones (unable to pass the first screen) from the third cycle, and a total of 16 base mutations (10 transitions and six transversions) were found. In addition, two base deletions and a gross re-arrangement at the 3′ side of the evolved region were observed in three random clones, likely expressing non-functional enzymes. These findings suggest that the selection process was successful in selecting functional top1s, and that about one

Table 2
Frequencies of amino acid mutations in selected and random clone sets at the third cycle

Mutation	Mutation frequency observed in clones of the third cycle		Expected frequency[a]
	Selected (6)	Random (8)	
Q633H	–[b]	1/8	–
L658Q	–	1/8	–
R663G	3/6	3/8	1/2
K669R	2/6	3/8	1/2
D677G	5/6	4/8	1/2
A678T	–	1/8	–
K681R	–	1/8	–
V683I	–	3/8	1/2
V703I	6/6	3/8	1/2
Q704L	1/6	–	–
N711I	–	1/8	–
K712R	1/6	2/8	1/2
T718A	–	1/8	–
N722Y	–	1/8	–
P726S	–	1/8	–
I728T	5/6	3/8	1/2
W736G	–	1/8	–
K742R	1/6	–	–
T747A	1/6	–	–

[a]Mutation frequency in clones of the second cycle used as parents for the third cycle. The domain border is at aa 712 (see also Fig. 1).
[b]Null frequency.

amino acid mutation per clone was, on average, introduced in the mutagenized cDNAs.

Among the 17 clones expressing a functional enzyme, the linker seemed to be somewhat more prone to bear amino acid mutations than the C-terminal domain (Table 2); that may be related to the highly conserved sequence of the latter. All the selected clones showed unique genotypes and the mean value of amino acid mutations per clone increased with the cycle number (Table 1). Interestingly, three amino acid changes were specifically associated with the selected phenotype, whereas two other mutations were apparently selected against (underlined mutations in Table 1). Even though the numbers are low and no statistical significance can be inferred, D677G, V703I and I728T changes, present in the two parent clones 5-II and 89-II, were found at a frequency higher than expected in the selected clone set of the third cycle, whereas they were close to the expected frequency among unselected clones (Table 2). V683I and K712R (of clone 89-II) were apparently selected against since one was absent and the other was present in only one clone of the selected set, and both were present among the unselected clone set at the expected frequency (Table 2). The sequence analyses thus suggest a role for D677G, V703I and I728T in determining the enhanced CPT sensitivity of top1s in JEL1-Δ*top1* cells, and showed that evolution of CPT-sensitive top1s followed a pathway that included recombination events, as well as the emergence of new mutations at each cycle (Table 1).

2.4. Mapping of mutations on top1 structure

The three mutations associated with the selected phenotype were localized on the crystal structure [11,13] and mapped to the non-conserved linker and C-terminal domains (Fig. 3), distant from the enzyme active site and a putative CPT receptor [11,13,26]. Asp 677 is part of the turn between the two α-helices at the tip of the linker; the Val 703 is towards the end of the second α-helix and makes contacts with hydrophobic residues (particularly Leu 617) of another α-helix (from residues 611 to 630) inserted between the linker and C-terminal domains in 3-D, but that was not evolved in the present work (Fig. 3). These two amino acids are not conserved, however the changes might influence protein structure: in particular, Val703Ile may change the hydrophobic linker surface

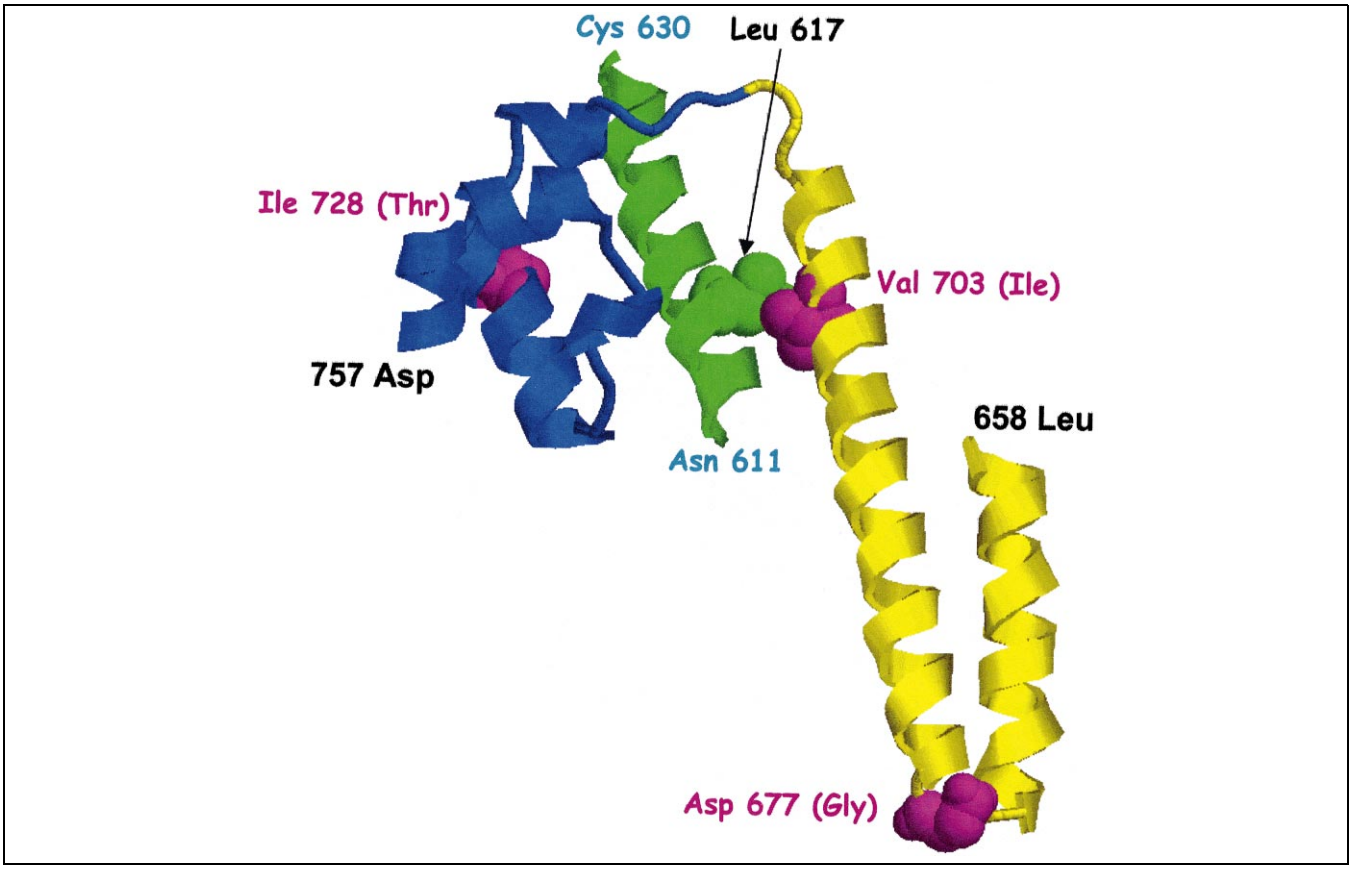

Fig. 3. Mapping of 5-II mutations on top1 crystal structure. The evolved region from Leu 658 to Asp 757 is shown in yellow (linker domain) and blue (C-terminal domain). The 5-II mutations, Val703Ile and Asp677Gly, are shown in magenta. In the crystal structure, Val 703 interacts with hydrophobic residues, including Leu 617, of the α-helix (green) from Asn 611 to Cys 630. The third mutation, Ile728Thr, associated with the evolved phenotype is also shown in magenta, and is located in the C-terminal domain. The drawing was made with RasMol (www.umass.edu/microbio/rasmol) using PDB entry 1A36.

that interacts with the 611–630 α-helix. The third mutation associated with the new phenotype, I728T, mapped instead of in the C-terminal domain to somewhat closer to the active tyrosine (Fig. 3). Therefore, the three mutations did not cluster close together in 3-D structure.

2.5. *Salt effects and CPT sensitivity of the best top1 mutants*

To investigate the molecular mechanisms of enhanced drug sensitivity, we have evaluated the biochemical properties of purified top1 mutants, including 5-II, 43-III, 50-III and 53-III. Since nuclear top1 content can affect CPT sensitivity [8,9,14,20], we firstly determined 5-II top1 contents in yeast cells after galactose induction, however no detectable difference was noted between wt and 5-II enzymes by Western blots (not shown). Purified 5-II and wt enzymes showed identical molecular weights (Fig. 4A), and analyses of DNA relaxation showed that the mutant had a comparable (though somewhat higher) specific activity as wt top1 (on average 7.2 and 4.9×10^5 U/mg, respectively). The effects of KCl were clearly different: for wt top1, DNA relaxation kinetics were much retarded by 270 mM KCl, whereas those of 5-II were minimally affected by high salt (Fig. 4B,C). The molecular phenotype was also evaluated by measuring the specific activities at 150 and 270 mM KCl of 5-II, as well as the best variants of the third cycle (Fig. 5). Supercoiled plasmid DNA was incubated with serial 1:2 dilutions of purified variants for a fixed time interval, and DNA relaxation was then evaluated by agarose gels. The specific activity of wt top1 was more than eight-fold decreased by 270 mM KCl, whereas the reduction of enzyme activity was of only two-fold for 5-II and 43-III, of two- to four-fold for 53-III, and of four-fold for 50-III (Fig. 5). Thus, the selected mutants are less affected than wt top1 by KCl concentration in the studied concentration range, and may likely have a higher DNA-binding affinity than wt enzyme. Moreover,

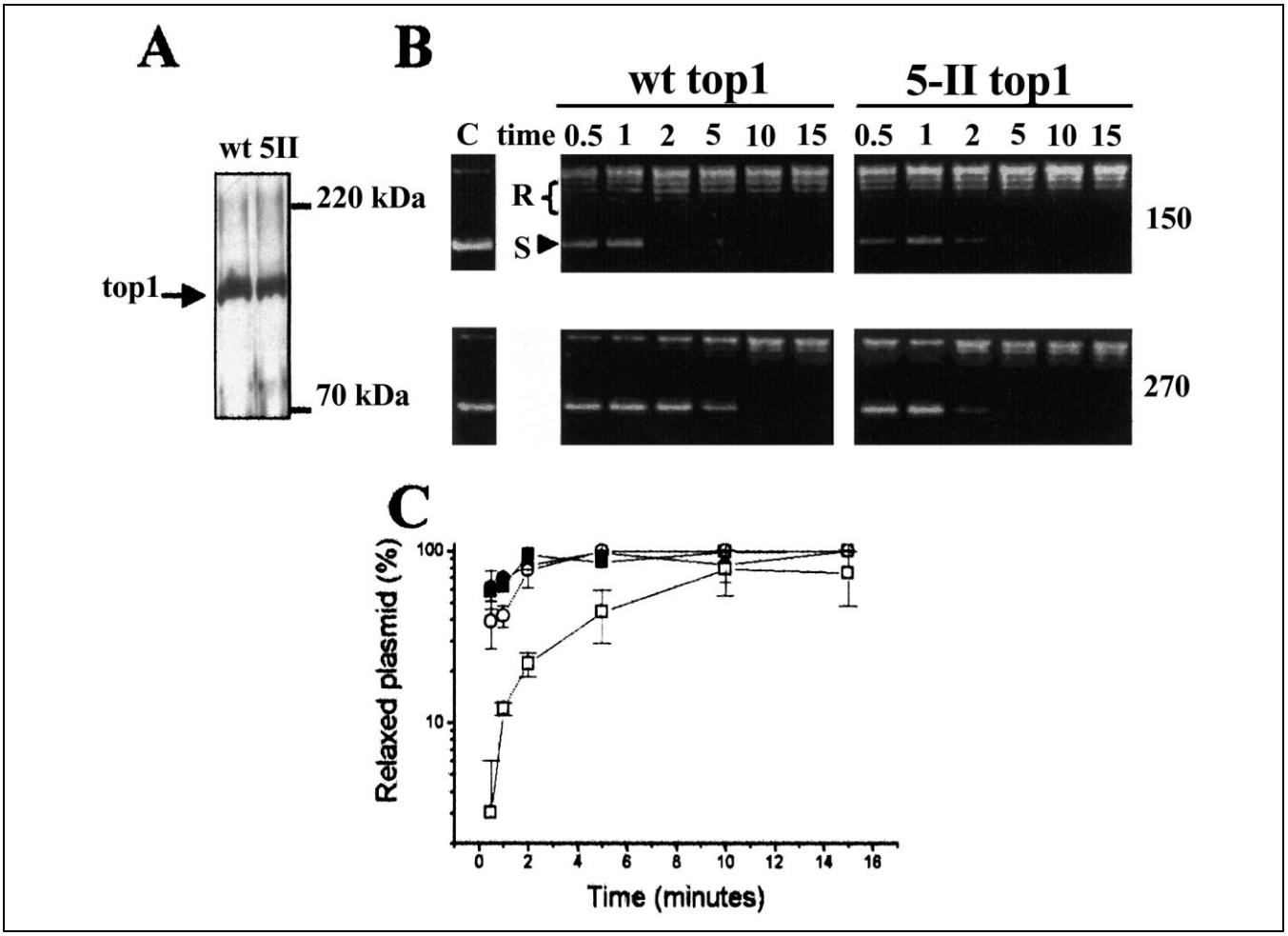

Fig. 4. Relaxation activity of 5-II mutant is less affected by high salt concentrations. (A) Purified wt and 5-II top1s were analyzed by Western blotting with a specific monoclonal antibody [21]. Molecular weight markers are indicated on the right. (B) Salt effects on relaxation kinetics of wt and 5-II top1. Supercoiled plasmid DNA was reacted with purified enzymes (25–32 U) for the time (min) indicated above the panel. Lane C, control DNA. On the left: S, supercoiled DNA; R, relaxed molecules. Enzymatic reactions were carried out at 150 and 270 mM KCl, top and bottom gels, respectively. (C) Gels similar to that of panel B were quantitatively analyzed to determine kinetic curves of DNA relaxation. Circles and squares correspond to 5-II and wt top1, respectively. Closed and open symbols correspond to 150 and 270 mM KCl, respectively.

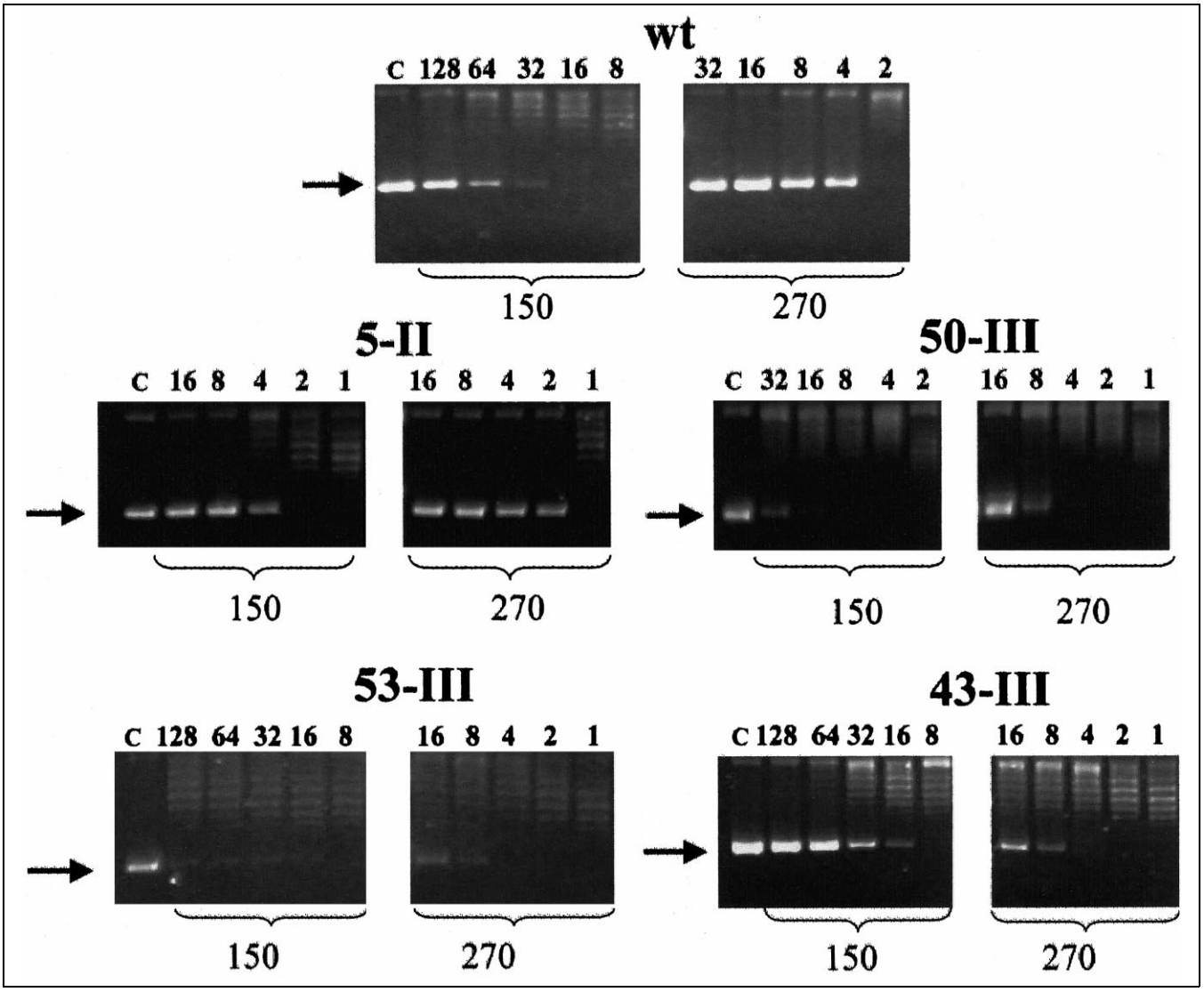

Fig. 5. Relaxation activity of top1 variants at diverse KCl concentrations. Supercoiled plasmid DNA (indicated by arrows) was reacted with purified enzymes (two-fold serial dilutions indicated above each lane) for 30 min at 37°C. Lanes C, control DNA. Enzymatic reactions were carried out at 150 and 270 mM KCl, as indicated at the bottom of gels. Representative gels are shown. At 150 and 270 mM KCl, respectively, full-relaxation activity was present at: 1:16/1:32 and 1:2 dilutions (at least eight-fold reduction) for wt top1; 1:2 and 1:1 dilutions (two-fold reduction) for 5-II; 1:16/1:8 and 1:4 dilutions (four-fold reduction) for 50-III; 1:8/1:16 and 1:4 dilutions (two- to four-fold reduction) for 53-III; 1:8 and 1:4 dilutions (two-fold reduction) for 43-III.

the findings indicate that the enzyme phenotype may vary among the selected top1 variants in terms of sensitivity to salt and DNA binding.

The 5-II variant was also investigated with a cleavage assay. KCl effects were determined on DNA cleavage activity of top1s by increasing the salt concentration in the reaction mix. In these experiments, we used amounts of wt and 5-II top1s that gave similar levels of DNA cuts at 150 mM KCl (Fig. 6). The DNA substrate, a 32-bp oligomer, contained a high-affinity binding site for top1 [23], and reacted with enzymes in the presence of CPT. Cleavage levels were markedly higher for 5-II than wt top1 by increasing KCl concentration (Fig. 6), showing that cleavage activity of 5-II was more resistant to salt. These results further supported that the mutant may have a higher DNA-binding activity, in agreement with DNA relaxation findings.

Moreover, wt and 5-II variant top1s were compared for CPT sensitivity with the same cleavage test at equal protein amounts at 150 mM KCl. CPT stimulated more cleavage with 5-II than wt top1 over the whole range of tested drug concentrations (Fig. 7), demonstrating that the 5-II mutant was more sensitive to CPT than wt enzyme. In particular, the increased drug sensitivity was marked (six- to seven-fold) at lower drug concentrations (Fig. 7). The 5-II amino acid changes, however, did not alter the site selectivity of DNA cleavage since patterns of cleavage intensity were identical for the two proteins in the studied DNA oligomer, as well as in longer fragments, such as simian virus 40 DNA (Fig. 7 and not shown).

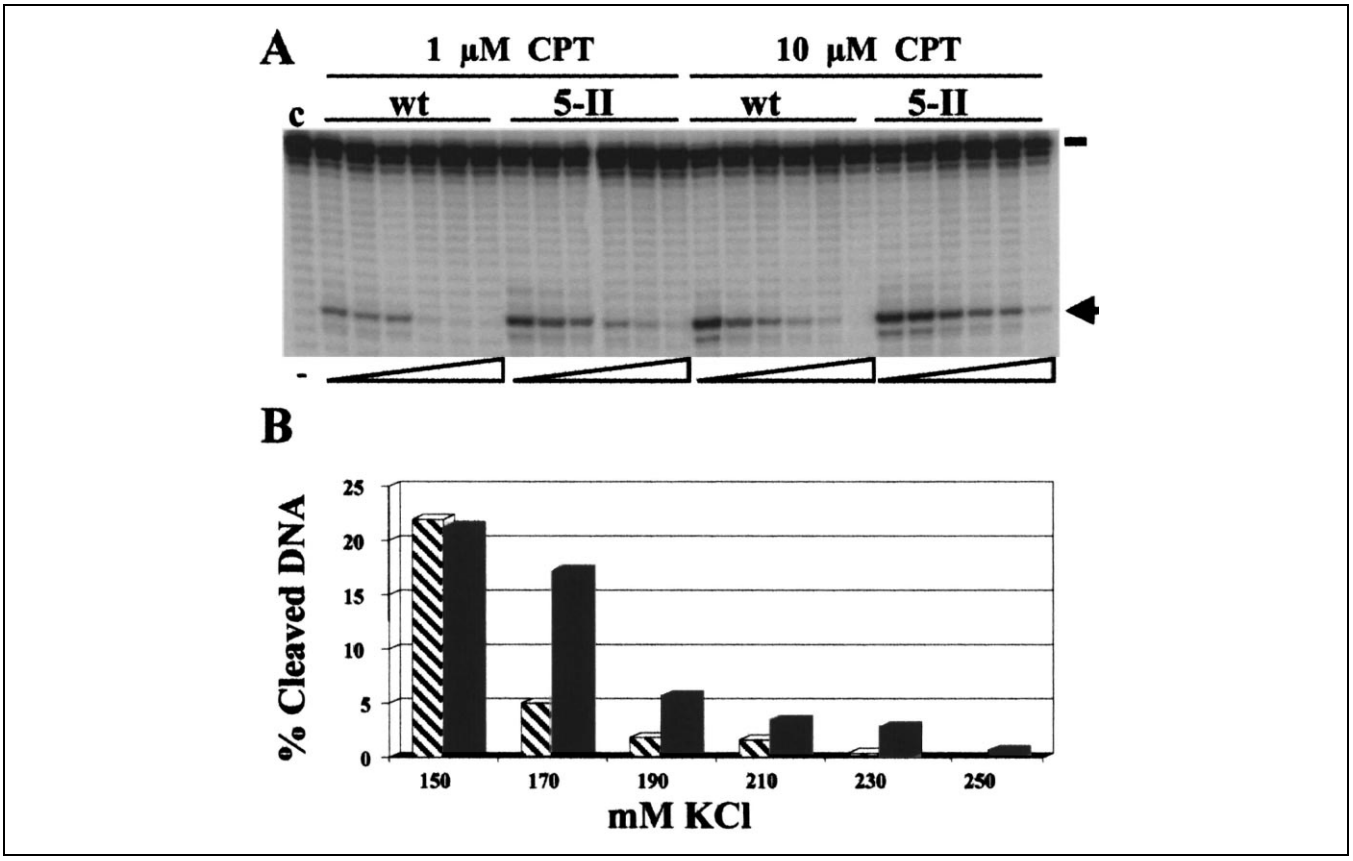

Fig. 6. Cleavage activity of 5-II mutant top1. (A) Salt effects on top1-promoted DNA cleavage. A DNA oligomer containing a high-affinity binding site for top1 [23] was radioactively 3′-end labeled and then reacted with wt or 5-II top1 with the indicated concentrations of CPT. The amounts of the two enzymes were added to obtain similar cleavage levels at 150 mM KCl. Reactions were terminated by SDS addition and DNA analyzed by sequencing gels. On the right, a bar indicates the full-length oligomer and an arrow indicates the cleaved band. Lane C, untreated DNA. At the bottom, each triangle indicates 150, 170, 190, 210, 230 and 250 mM KCl, from left to right. (B) Cleavage levels at 10 μM CPT versus KCl concentration, determined from gels similar to that of panel A. Standard errors of the mean are within 23% of indicated values. Dashed and black bars correspond to wt and 5-II top1s, respectively.

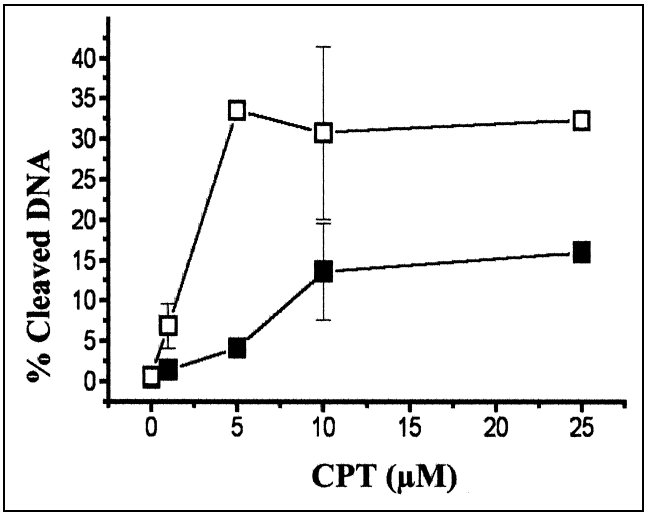

Fig. 7. CPT sensitivity of wt and 5-II top1s. A DNA oligomer containing a high-affinity binding site for top1 [23] was radioactively 3′-end labeled, reacted with 50–70 ng of wt or 5-II top1 in the presence of different CPT concentrations for 30 min at 37°C, and then processed as in Fig. 5. Cleavage levels were then measured by phosphorimager analyses. Open and closed squares are 5-II and wt top1s, respectively.

2.6. *Effects of expression of 5-II top1 in a repair-deficient yeast strain*

The 5-II mutant was also studied in JN2-134*top1-1* yeast strain that is defective for homologous recombinational DNA repair, and is thus more sensitive to topoisomerase poisons [10,20]. In agreement with the data on the JEL1 strain, the evolved top1 conferred enhanced CPT sensitivity to JN2-134*top1-1* cells since the drug $ID_{5\%}$ was almost three-fold lower for 5-II (Table 3).

We must also note that the cloning efficiency of JN2-134*top1-1* cells was reduced by both proteins under our experimental conditions, indicating a slight toxicity of human top1 for repair-deficient yeast cells. In contrast, the cloning efficiency of the repair-competent JEL1-Δ*top1* strain was minimally, if any, affected by wt and 5-II enzymes (Table 3). After galactose activation of enzyme expression, JEL1-Δ*top1* cell growth was not different among wt, 5-II, 51-III, 50-III and 43-III top1s (not shown), documenting that the evolved human proteins were not toxic to repair-proficient yeast cells even at high expression levels. Interestingly, the 5-II enzyme was two-fold more ef-

Table 3
Effects of 5-II mutations on yeast cloning efficiency and CPT sensitivity in a *rad52*$^-$ strain[a]

Enzyme	Cloning efficiency (%)		CPT cytotoxicity in JN2-134*top1-1*
	JEL1-Δ*top1*	JN2-134*top1-1*	$ID_{5\%}$ (μM)[b]
no top1	100	100	nd
wt top1	79	33	0.31
5-II top1	90	16	0.12

[a]Top1 cDNAs were expressed from the 2-μ-based pEZ-hTop1 plasmid, and the assays were performed in 2% glucose. JN2-134*top1-1* is *rad52*$^-$, thus lacking the homologous recombinational repair pathway, and JEL1-Δ*top1* is a repair-proficient strain [20].
[b]Cytotoxicity was measured with a clonogenic test. $ID_{5\%}$ values are CPT concentrations reducing colony formation to 5% of respective untreated cells. nd, much higher than the highest dose used (10 μM).

fective than wt top1 in reducing the cell cloning efficiency of repair-deficient JN2-134*top1-1* cells (Table 3). Altogether, the findings thus indicated that 5-II likely promoted higher levels of genomic cleavage than wt top1, in agreement with observations on purified proteins (Figs. 6 and 7).

3. Discussion

Our results demonstrate that directed evolution of a eukaryotic DNA-binding protein, human top1, can be achieved in yeast by DNA shuffling. Novel top1 variants have been evolved that are more efficiently transformed into lethal toxins by CPT. Although clones of the first cycle gave a weak phenotype, we could get a more marked phenotype at the second (5-II) and third (43-III) cycles. At each cycle, selected clones of the previous one were used as parent clones, and analyses of the genotypes of all selected clones (Table 1) clearly suggest that the combination of recombination events and novel mutations generated the new selected clones, in agreement with previous approaches of DNA shuffling [2–6].

Top1 mutants with a high toxicity to cells were known already, and they showed a much greater tendency to damage cellular DNA [27,28]. However, those mutants are toxic by themselves, whereas our evolved top1s remain fully and normally functional, and are not lethal by themselves in DNA-repair proficient yeast cells. That reflects our selection process: the selection pressure during the evolution cycles was not only for increased sensitivity to the drug (CPT), but also for cell growth. Thus, in order to be selectable, the plasmid-encoded top1 needed to be not harmful to yeast cells without drug.

Many CPT-resistant top1 variants were described [9,10,29–31] providing information on structure–function relationships of the enzyme. To our knowledge, our work is the first reporting on human top1 mutants that are instead more sensitive to CPT. Molecular analyses of the 5-II variant, as well as of the best three variants (Table 1), show that the evolved top1s likely have a higher apparent DNA-binding affinity. The three best variants (50-III, 53-III, and 43-III) have four or six mutations, but each has a unique mutation pattern. All the mutations are distant from the putative CPT receptor site [9,11,13,15,25,26], likely indicating that they do not directly affect CPT interactions with top1–DNA complexes. Rather, overall conformational dynamics of the protein may be altered in such a way to increase apparent DNA binding and CPT sensitivity.

Three mutations, D677G, V703I, and I728T, were found to be associated with selected phenotype. D677G and V703I (the only mutations of 5-II mutant) are located in the linker domain, which is not conserved in eukaryotic top1s [8,9] and is even absent in some instances, such as the vaccinia virus top1 [32]. Based on the crystal structure, it has been proposed that the linker domain may function as a brake of the rotation of the broken strand around the uncut strand during the catalytic cycle [11,13]. An alteration of this function may lead to a higher number of strand rotations per catalytic cycle and may affect enzyme processivity and apparent DNA binding. We have indeed presented findings suggesting such biochemical differences between the 5-II variant and wt top1. Moreover, the best variants of the third cycle (43-III, 50-III, and 53-III) were less sensitive to KCl than wt top1, indicating that a consistent phenotype was selected. However, whether the altered linker structures of our variants result in a diminished break–rejoining activity of top1 needs to be established with a suicide DNA substrate.

The contribution of each mutation to the evolved top1 phenotype remains to be established. However, the DNA shuffling technology often results in the selection of proteins with several mutations. In our case, it is intriguing to note that the Thr729Ala mutation (adjacent to I728T, Table 1) was reported to confer CPT resistance [31]. A single-mutant top1 with a Thr residue at 728 was only slightly different, if any, from wt top1 in cytotoxicity assays (unpublished data). Since DNA shuffling is a powerful tool to select combinations of mutations that affect protein phenotypes weakly when alone and markedly when combined [4,5,33], the I728T mutation might contribute to the evolved phenotype only when with other mutations. Our best variants, 50-III, 53-III and 43-III have distinct mutation patterns, indicating that, in these cases, enhanced CPT sensitivity may likely be determined cooperatively by amino acid changes.

An increase of DNA binding and/or an altered break–rejoining activity can likely enhance the stability of DNA–drug–top1 complexes. Thus, CPT action is favored and the enzyme becomes more CPT-sensitive. Consistently, a common mechanism of drug resistance of top1 is a decrease in DNA binding [9,25,28]. It is also interesting to note that the vaccinia top1, which lacks a linker domain, is very resistant to CPT [28,32]. The influence of the linker domain on CPT sensitivity has been investigated recently

by deleting large protein portions [34,35]. A first work on reconstituted human top1 showed that the lack of the whole linker domain decreased DNA binding and cleavage levels with CPT [34]. Another report from the same group [35] described a deletion of residues 660–688 that corresponds essentially to the first quarter of the evolved region of this work (Fig. 3). Such a deleted top1 could be purified as monomeric and dimeric enzyme forms. Interestingly, the monomeric form was insensitive to CPT and distributive in relaxation assays, whereas the dimeric form was CPT-sensitive and -processive. The similarity of dimeric form to wt top1 was discussed by the authors in terms of some structural features of the dimer that can surrogate a full-functional linker [35]. Our findings on evolved top1s demonstrate that amino acid mutations in the linker domain can affect enzyme processivity and CPT sensitivity, and are thus in agreement with the above results. Our data however, do not rule out the possibility that some mutations of the evolved enzymes can alter other molecular mechanisms as well.

Here, we evolved a relatively short sequence (100 aa) of human top1, however, the procedure could be applied to longer sequences as well, thus evolving several domains at the same time. The design of CPT-hypersensitive top1 mutants is a step towards the development of a nuclear DNA-damaging toxin that might be used to specifically destroy cancer cells or pathological microbes in a suicide gene-therapy approach [7]. Moreover, drug-hypersensitive top1s can be valuable tools to improve the sensitivity of assays used in current screening programs to find novel anticancer compounds.

4. Materials and methods

4.1. Materials

The 2-μ-based pEZ-2ThTop1 plasmid bears a fused GST domain to wt human top1 under the control of a galactose-inducible hybrid promoter [7]. JEL1-Δ*top1* [19] and JN2-134*top1-1* [20] yeast strains, which lack the endogenous TOP1 gene, were provided by P. Benedetti (Padova University, Padua, Italy). A monoclonal antibody against human top1 was provided by I. Bronstein (York University, York, UK) [21]. CPT was purchased from Sigma, St. Louis, MO, USA.

4.2. Library of shuffled top1 cDNA

The full-length human top1 was fused to the GSH-binding domain of *Schistosoma japonicum* GST enzyme and cloned into pEMBLyex4 plasmid, resulting in pEZ-hTop1 plasmid as described already [7]. The evolved fragment was from nucleotides 2186 to 2483 (297 bp) of top1 cDNA, corresponding to the linker and C-terminal protein domains (Fig. 1B). Two primers, sere44 and sere11, external to this region, were designed to amplify a 525-bp fragment (from 2072 to 2597 bases) that shares terminal sequences of about 115 bp with pEZ-hTop1 DNA digested with *Nhe*I and *Bst*XI endonucleases (Fig. 1B). Thus, steps of evolution cycles were the following: (A) Error-prone PCR to amplify the 525-bp fragment was performed as described [22]: reactions were in 67 mM Tris–HCl, pH 8.8, 6.1 mM $MgCl_2$, 1 mM each dNTP, 16.6 mM ammonium sulfate, 6.7 μM EDTA, 10 mM β-mercaptoethanol, 10% dimethyl sulfoxide, 48 nM each primer, 30 ng of sere11 and sere44, 5 U Taq polymerase (Perkin-Elmer) for 30 cycles of 1/1/2 min at 95/42/72°C. (B) Random fragmentation of PCR products [4,5]: 15 μg of DNA was incubated in 50 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, with DNase I 0.3–0.4 U in 70 μl for 1 min at 25°C and stopped with EDTA and SDS. (C) After gel-purification of fragments, DNA was re-assembled [4,5] by PCR without primers in 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 2.2 mM $MgCl_2$, 0.2 mM each dNTP, Triton X-100 0.1%, and 2.5 U of Taq polymerase (Promega) for 45 cycles of 0.5/0.5/0.5 min at 94/54/72°C. (D) The 525-bp fragment was PCR-amplified using sere44 and sere11 with standard PCR conditions. (E) Cloning of 525-bp fragments into pEZ-2ThTop1 by homologous recombination in JEL1-Δ*top1* yeast. Vector DNA was digested with *Bst*X1 and *Nhe*I, and fragments were purified with gel electrophoresis. Then, yeast cells were transformed with gapped plasmid and shuffled PCR product in a $5–10\times10^3$ molar excess. Exponentially-growing cells were washed and resuspended in 0.1 M Li-acetate, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA. 2 μg of DNA with 100 μg of salmon sperm DNA were added to cells in 40% PEG 4000, 0.1 M Li-acetate, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA. After 1 h of incubation at 30°C, cells were shocked at 42°C for 1 min, centrifuged, washed, and plated to have 100–180 colonies/plate. (F) Selection of clones for increased cell sensitivity to CPT by replica plates in synthetic complete (SC)-uracil medium.

4.3. Selection and sequencing of CPT-hypersensitive clones

Libraries of $0.8–1\times10^4$ clones were prepared, and clones were initially selected by replica plating for enhanced sensitivity to 10 (first and second cycles) and 3 μM (third cycle) of CPT. This step selected 65–130 clones. A second screen was then performed: 1:5 serial dilutions of cells of selected clones were spotted onto plates containing SC-uracil medium without or with 3 or 10 μM CPT. With the second screen we usually selected 15–17 clones. Then, a third screen was performed with an in-liquid assay (see below), which reduced the selected clones to 6–8. The plasmids were recovered from final clones, and re-transformed into fresh JEL1-Δ*top1* cells. Then, new colonies were recovered and tested with the in-liquid assay. At each step, clones showing a higher sensitivity than wt top1 were selected and proceeded to the next one. CPT lactone form was always used throughout this work.

Cell killing assays were: (A) In-liquid cytotoxicity test. Exponentially-growing cells were diluted to 0.5 OD_{600nm} in SC-uracil medium and incubated with different CPT concentrations for 18 h at 30°C. Cell growth inhibition was then tested by measuring the OD_{600nm} of cell cultures. (B) Clonogenic test. CPT sensitivity of selected top1s was determined in vivo by a colony-forming inhibition assay. Exponentially-growing cells were treated with different CPT concentrations for 18 h at 30°C, then appropriate dilutions of cultures were plated in duplicate in SC-uracil medium and colonies were allowed to grow for 3–4 days. Cell survival was determined as ratios of the numbers of colonies formed by treated and control cells.

Sequencing of evolved regions was performed on an automated

fluorescent ABI PRISM 377 DNA sequencer using the T7 Sequencing Kit (Pharmacia).

4.4. Biochemical assays

Evolved and wt top1s were purified from yeast nuclear extracts with ammonium sulfate precipitation, phosphocellulose chromatography and affinity chromatography as described [7]. DNA relaxation and cleavage assays, Western analyses, and ^{32}P-3′-end labeling of DNA oligomers were performed as extensively described already [7]. Briefly, negatively-supercoiled pBR322 plasmid DNA (250 ng) was incubated with 25–32 U of enzyme in 20 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 0.1 mM dithiothreitol, 24.5 mg/ml bovine serum albumin, 4.5% glycerol, and 150 or 270 mM KCl for the indicated time at 37°C. After gel electrophoresis, stained gels were analyzed with Bio-Rad Gel Doc 1000, and relaxed DNA determined with Bio-Rad Molecular Analyst software. A 32-bp DNA oligomer containing a high-affinity binding site for top1 [23] was used as the substrate in cleavage assays. DNA cleavage was analyzed by 17% polyacrylamide sequencing gels, and levels were determined by PhosphorImager (Molecular Dynamics) analyses as reported already [7].

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