DNA topoisomerase I (Top1p) catalyzes the relaxation of supercoiled DNA through the transient cleavage of one strand of a DNA duplex and is fundamental to processes such as replication, recombination, and transcription (1–4). The three-dimensional structure of reconstituted and N-terminal truncated versions of human topoisomerase I (hTop1p) in complex with a 22-bp DNA molecule have shown that the enzyme is organized in multiple domains that “clamp” around the DNA molecule (5, 6). Changes in DNA topology are achieved by introducing a transient break in the phosphodiester bond of one strand in the duplex DNA. The phosphodiester bond energy is preserved during catalysis through the formation of a transient covalent phosphotyrosine bond between the catalytic Tyr-723 and the 3’ end of the broken DNA strand. DNA relaxation has been proposed to proceed via a “controlled rotation” in which the covalently bound enzyme holds one end of the DNA duplex and allows the end downstream of the cleavage site to rotate around the remaining phosphodiester bond (6). Eukaryotic Top1p is the cellular target of the anti-tumor drug camptothecin (CPT), which reversibly stabilizes the cleavable intermediate complex formed in the catalytic cycle of the enzyme (1, 2, 7).

Recently Staker et al. (8) have solved the x-ray crystal structure of hTop1p covalently joined to double-stranded DNA and bound to the CPT analog Topotecan (8). This structure revealed that the drug molecule intercalates between upstream (−1) and downstream (+1) bp, displacing the downstream DNA and thus preventing the religation of the cleaved strand. The structure helps to clarify the role of several, but not all, mutant residues that produce a CPT-resistant enzyme. In particular the structure permits to explain the CPT resistance for mutations involving residues that interact directly with the drug or that would alter the interaction with DNA. However it is not able to explain the CPT resistance for point mutations such as Ala-653 (9), Glu-418 (10), and Thr-729 (11), which involve residues that are too distant to contact the drug directly. Among them the Ala-653 mutation in proline (hTop1A653P), residing in the linker domain, is intriguing, because it has been shown that the linker region is required for maximum sensitivity to CPT (12). The linker region is in fact supposed to be involved in the relaxation reaction, possibly acting as a “brake” during the rotation of the DNA, downstream the cleavage site (12).

In this paper, we have investigated the effect of the single A653P mutation through experimental and simulative approaches to clarify the role of the linker domain. The data indicate that the single mutant displays an increased religation rate and a high CPT resistance. Molecular dynamics (MD) simulation reveals that the substitution renders the linker more flexible, providing a structural dynamical explanation for the experimental results.

**EXPERIMENTAL PROCEDURES**

**Materials, Yeast Strains, and Plasmids—**Camptothecin (Sigma) was dissolved in Me2SO to a final concentration of 4 mg/ml and stored at −20 °C. MAb monoclonal antibody was purchased from Sigma. Scecharamyces cerevisiae strains JN2–134 (MATa rad52::LEU2, trpl, ade2−1, his7, ura3−3, is1, top1, leu2) and EKY3 (ura3−3, his3200,leu221, trpl36, top1::TRP1, MATa) were described previously (13, 14). Plasmids YEpGal1−hTOP1 and YcpGal1−hTOP1 in which the human Top1 is expressed under the galactose-inducible promoter in a multicopy plasmid and single copy plasmid, respectively, have been described (13, 15). pBlueAK3−1 DNA contains a high affinity Top1 cleavage site (16). Randomly mutagenized htop1 pools were produced as described.
and YEpGAL1 were grown to an molar excess of CP25 over CL14 by heating to 95 °C in 60 min according to Stewart et al. (12). The duplex substrate was generated as described above. The suicide cleavage reactions were carried out by incubating 20 nM of the duplex with an excess of enzyme in 20 mM Tris (pH 7.5), 0.1 mM Na2EDTA, 10 mM MgCl2, 50 µg/ml acetylated BSA, and 100 mM KCl at 37 °C in a final volume of 50 µl as described in Yang et al. (35). A 5-µl sample of the reaction mixture before the addition of the protein was removed and used as the zero time point. At various time points 5-µl aliquots were removed and stopped with 0.5% SDS. After ethanol precipitation samples were resuspended in 5 µl of 1 mg/ml trypsin and incubated at 37 °C for 30 min. Samples were analyzed by denaturing urea/polyacrylamide gel electrophoresis. The percentage of the remaining covalent complex (cleavage 1) was determined by PhosphoImager and ImageQuant software and normalized on the total amount of radioactivity in each lane. The recombination rate (kh) was determined by fitting the data, up to 4 min, to the equation ln (%remaining cleavable complex) = 4.605 – kθ t (12, 20, 21).

**Kinetics of Cleavage Using Oligonucleotide Substrate**—The duplex substrate was generated as described above. The suicide cleavage reactions were carried out by incubating 20 nM of the duplex with an excess of enzyme in 20 mM Tris (pH 7.5), 0.1 mM Na2EDTA, 10 mM MgCl2, 50 µg/ml acetylated BSA, and 100 mM KCl at 37 °C in a final volume of 50 µl as described in Yang et al. (35). A 5-µl sample of the reaction mixture before the addition of the protein was removed and used as the zero time point. At various time points 5-µl aliquots were removed and stopped with 0.5% SDS. After ethanol precipitation samples were resuspended in 5 µl of 1 mg/ml trypsin and incubated at 37 °C for 30 min. Samples were analyzed by denaturing urea/polyacrylamide gel electrophoresis. The percentage of cleavage 1 was determined by PhosphoImager and ImageQuant software and normalized on the total amount of radioactivity in each lane.

**MD Simulation**—The starting coordinates of the linker domain (residues 636–712) were extracted from those of DNA topoisomerase I obtained from x-ray diffraction (Protein Data Bank entry 1A36) (5). The five residues constituting the loop region that connects the linker to the core domain (residues 636–640), which are lacking in the Protein Data Bank structure because of thermal fluctuation, were added to the linker domain by molecular modeling. The spatial environment of each new residue was checked for close contact or overlap with neighboring residues, and stereochemical regularization of the structures was obtained by the Powell minimization method implemented in the SYBYL program (Tripos Inc., St. Louis, MO). The residue Ala-653 was changed to Gln to create a flexible linker, and to stabilize the structure of the substituted residue were repaired and adapted to the introduced stereochemical constraint. Model building and molecular mechanics were carried out on a Silicon Graphics O2 R5000 SC using the version 6.2 of the SYBYL program. The system topology was built using the AMBER leap module and the AMBER95 all atom force field (22). The system was immersed in periodic boundary conditions, using a cutoff radius of 9 Å for the non-bonded interactions and updating the neighbor list every 10 steps. The electrostatic interactions were calculated with the Particle Mesh Ewald method (24, 25). The SHAKE algorithm (26) was used to constrain all bond lengths involving hydrogen atoms. Optimization and relaxation of solvent and ions were initially performed keeping the solute atoms constrained to their initial position with decreasing force constants of 500, 25, 15, and 5 kcal/mol Å, whereas the linker N-terminal and C-terminal residues (Pro-636 and Lys-712) were kept constrained on their initial positions for the whole simulation under the SHAKE conditions. Thereafter the system was optimized without any constraint, simulated for 1 ps at 100 K, and then warmed up to 300 K and equilibrated over 60 ps. A 1-ns simulation was carried out at constant temperature of 300 K using the method of Berendsen et al. (27), at a constant pressure of one bar with a 2-fs time step, on a four-processors UNIX server Origin 200 SGI. Pressure and temperature were 0.5 ps. The last nanosecond of trajectories were saved every 100 steps (0.2 ps) for analysis. To better quantify the mobility of the mutated linker domain we have superimposed the MD conformations over the three-dimensional structure of the DNA-Top1 covalent complex (Protein Data Bank entry 1A36). Deviation of the linker domain from the starting structure was evaluated by measuring the dihedral angle between its geometric points: a, the geometric center (centroid) between nucleotides Thr-22 and Ala-101; b, the centroid between nucleotides Ala-16 and Thr-107; c, the centroid between residues Pro-636 and Lys-712; d, the centroid between residues Leu-658 and Leu-94.
RESULTS

The Catalytic Active Mutant hTop1A653P Is Resistant to CPT—We have previously described in Fiorani et al. (9) a yeast S. cerevisiae genetic screen for catalytically active CPT-resistant hTop1 mutants. Briefly JN2–134 (rad52/H9004) cells tolerate galactose induced hTop1 expression from a single copy vector, but exhibit decreased viability when the enzyme is expressed from pGAL1 on a multicopy vector. The leaky expression from the same multicopy vector is sufficient to render JN2–134 sensitive to CPT. Thus catalytically active CPT-resistant hTop1 mutants may be selected by replica plating CPT-resistant colonies on galactose plates and identifying the inviable cells. Fig. 1 (panel A) shows the result of the yeast screen for the hTOP1 and the htop1A653P and htop1G363C mutants. This latter mutant was used as control, because it was already shown to be CPT-resistant (17). The results indicate that substitution of Ala-653 with proline confers CPT resistance, and the mutant retains sufficient catalytic activity in vivo to kill yeast strain JN2–134 when is overexpressed in the presence of galactose.

Indication of CPT resistance of the mutant was also obtained from a cell viability assay in liquid culture carried out as described under "Experimental Procedures." As shown in the graph of Fig. 1 (panel B) cells expressing the htop1A653P mutant in the presence of 50 μM CPT exhibit a more than 100-fold resistance, when compared with the cells expressing hTOP1 in the same conditions.

To better characterize the catalytic activity of the mutant and to exclude that the cellular resistance to CPT is because of a reduction in catalytic activity a functional assay of hTop1A653Pp was carried out in vitro. Equal protein concentrations of hTop1p and hTop1A653Pp were serially diluted and incubated with supercoiled plasmid DNA to assess their ability to relax a supercoiled plasmid. As illustrated in Fig. 1 (panel C) the mutant hTop1A653Pp is active in vitro although, when compared with hTop1p under standard conditions, a 5- to 10-fold reduction in activity can be estimated.

The catalytic activity of hTop1A653Pp is more sensitive to high ionic strength than the wild type enzyme. In fact the activity of hTop1A653Pp drops when the concentration of KCl is higher than 150 mM, whereas hTop1p is able to relax supercoiled DNA up to 300 mM KCl (data not shown), indicating that the single mutation on the linker domain is able to perturb the electrostatic interaction between the enzyme and DNA. Similar ionic strength sensitivity has been reported for the two reconstructed enzymes, topo58/12, in which the linker domain is not covalent bound to the core subdomain III, and topo58/6.3, in which the linker domain is completely deleted (28).

Fig. 1. htop1A653P is resistant to CPT in vivo. Panel A, exponentially growing cells in dextrose, transformed with YEpGAL1-hTOP1, YEpGAL1-htop1A653P, YEpGAL1-htop1G363C, or YEpGAL1 (Vector) were serially 10-fold diluted starting from A₅₉₀ = 0.3; 5 μl was spotted onto selective media in the presence of dextrose (DEX) or in the presence of dextrose and 5 μg/ml CPT (DEX Cpt 5 μg/ml) or induced with galactose (GAL). Panel B, exponentially growing cells in dextrose transformed with YCpGAL1-hTOP1, YCpGAL1-htop1A653P, or YCpGAL1 (Vector) were diluted 1:100 into selective media containing 2% raffinose. After induction with 2% galactose cells were treated with 50 μM CPT or 1% Me₂SO. At various time points aliquots were serially diluted and plated onto selective media containing 2% dextrose. The number of colonies was counted (relative to that obtained at time 0) and plotted against time for the hTop1 (solid line), hTop1A653P (dotted line), and vector (dashed line) in the absence (open symbols) or presence (filled symbols) of 50 μM CPT. Panel C, partially purified hTop1p (lanes 1–3) and hTop1A653Pp (lanes 4–6) were normalized to the same amount of Top1 protein and 10-fold serially diluted in reaction buffer. 1 μl was assayed for their ability to relax 0.5 μg of supercoiled plasmid DNA as described under "Experimental Procedures." C, untreated DNA.
creasing protein concentrations of hTop1p and hTop1A653Pp in the presence of CPT. The reactions were stopped with 0.5% SDS, and the products were treated with proteinase K and separated by urea/polyacrylamide gel electrophoresis. The cleaved DNA fragments were resolved in a denaturing polyacrylamide gel (Fig. 2). Increasing protein concentrations of hTop1 and hTop1A653P proteins have been incubated without CPT (lanes 1–4 and 9–12) or presence (lanes 5–8 and 13–16) of 100 μM CPT. The reactions were stopped with 0.5% SDS, and the products were treated with proteinase K and separated by urea/polyacrylamide gel electrophoresis. The arrow indicates the preferential cleavage of hTop1p. C, untreated DNA.

Effect of CPT on the Cleavage-religation Equilibrium—To assess the level and mechanism of camptothecin resistance displayed by the hTop1A653Pp, the stability of the covalent DNA-enzyme complex has been analyzed. Increasing concentrations of hTop1p and hTop1A653Pp have been incubated with a 3' end-labeled DNA fragment, in the presence or absence of 100 μM CPT. Following incubation at 37 °C for 30 min, the cleavable complexes have been trapped with SDS/proteinase K treatment, and the cleaved DNA fragments were resolved in a denaturing polyacrylamide gel (Fig. 2). Increasing protein concentrations have been used to be sure that the entire DNA is bound to the enzyme so that the amount of cleavage can be taken as an approximate measure of the cleavage-religation equilibrium (12). When wild type hTop1p is incubated without CPT (lanes 1–4), a very small level of cleavage of the labeled DNA strand is observed at the preferred cleavage site, as indicated by the arrow in Fig. 2 (29). When hTop1p is exposed to CPT, a dramatic increase in cleaved DNA fragments is observed (lanes 5–8) indicating that the equilibrium is shifted toward cleavage. In the case of hTop1A653Pp in the absence of the drug (lanes 9–12) no cleavage of the labeled DNA strand is observed, indicating that the mutant enzyme is so fast in the religation step that no cleavage can be detected. The low level of cleavage observed in the presence of the CPT (lanes 13–16) confirms this latter hypothesis indicating that the equilibrium is still shifted toward religation. However some cleavage occurs in the presence of CPT for the hTop1A653Pp, providing a direct evidence for CPT binding to the mutant and indicating that the cleavage-religation equilibrium of the hTop1A653Pp is close to that of the wild type enzyme in the absence of CPT.

Analysis of the Religation Rate Using a Suicide Substrate—To understand how CPT affects the cleavage-religation equilibrium in the case of the hTop1A653Pp an experiment that directly measures the religation rate has been performed. The oligonucleotide substrate CL14 (5'-GAAAAAGACTTAG-3'), containing a preferred cleavage site for hTop1p, was radio-labeled at its 5' end and annealed to the CP25 complementary strand (5'-TAAAAATTTTCTAGAGTCTTTTTT-3') to generate a suicide cleavage substrate (12). This suicide cleavage substrate has been incubated with an excess of hTop1 and hTop1A653P enzymes to allow suicide cleavage to proceed to completion. 200-fold molar excess of R11 oligonucleotide (5'-AGAAAAATT TTT-3') was added in the absence and presence of CPT, and aliquots have been removed and analyzed at different times on urea-polyacrylamide gel as described under "Experimental Procedures."

A typical experiment in the absence and presence of CPT is shown in Fig. 3. The percentage of the remaining covalent complex (cleavage 1) was determined and plotted in Fig. 4. The data show a 3-fold increase in the religation rate $k_r$ for the hTop1A653Pp compared with the wild type enzyme, in the absence of CPT. The presence of CPT, as expected, dramatically decreases the religation rate in the wild type protein, whereas an analogous effect is not observed in the case of the hTop1A653Pp, confirming its CPT resistance.

Analysis of the Cleavage Rate Using a Suicide Substrate—A suicide cleavage substrate, identical to the one described in the religation reaction, has been incubated with an excess of hTop1p and hTop1A653Pp in a time course experiment. The cleaved DNA fragments were resolved in a denaturing polyacrylamide gel (Fig. 5, panel A). The amount of cleaved DNA for hTop1A653Pp and hTop1p was determined as described under "Experimental Procedures," normalized to the plateau value for the hTop1p, and plotted against the incubation time (Fig. 5, panel B). At 30 s the level of DNA cleavage for hTop1p and the hTop1A653P enzymes is 70 and 30% of the final value respectively, indicating an almost 2-fold decrease of the cleavage rate ($k_c$) of the mutant compared with the wild type protein.

Modeling—To identify the structural dynamical properties associated with the A653P mutation responsible of the peculiar functional properties, 1 ns of classical molecular dynamics simulation for the wild type and mutated linker has been carried out. As stated under “Experimental Procedures,” the trajectories have been generated in a fully hydrated environment anchoring the N and C termini of the linker to simulate its binding to the large remaining part of the protein. Analysis of the 1-ns trajectories of the wild type and mutated linker domain indicate that the overall structure is maintained throughout the simulation time in both cases. In particular the secondary structure of each amino acid residue, analyzed with the DSSP program (30) and plotted as a function of time in Fig. 6, is fully maintained. Some loss of secondary structure is only observed at the N-terminal region, with a slightly larger extent in the case of the mutated linker domain. This result indicates that the non-covalent interactions occurring in the 636–712
The linker segment are sufficient to maintain a stable structure also once the segment is separated from the whole Top1 protein, i.e. the continuous set of hydrogen bonds occurring in the two α-helices have a crucial role in maintaining the linker structure. This is true also for the A653P mutation, where the breakage of a hydrogen bond because of the proline insertion is not enough to perturb the overall stability of the linker and only induces some secondary structure loss at the N-terminal region.

The per-residue root mean square fluctuations display a different behavior for the wild type and mutated linker domain. In both cases a gradient of increasing fluctuations quite symmetric with respect to the center of the linker region is observed, the maximum being localized on the residues forming the turn connecting the two helices (Fig. 7). The absolute value of the fluctuations is much larger in the mutated linker domain, which also shows another maximum of fluctuation in the N-terminal amino acid residues bonded to the core domain, not observed in the native linker (Fig. 7). These results indicate that the linker domain of the mutant samples a larger confor-
results. This oscillation reaches much larger values in the mutated domain when compared with the wild type and can be measured by comparing the dihedral angle value $a, b, c, d$ (as described under “Experimental Procedures”) during the simulation. The wild type linker domain, in fact, visits a conformational space around a dihedral angle of 52 degrees close to the starting conformation (Fig. 8B). The mutated linker, on the contrary, progressively changes its orientation until a final dihedral angle of 26 degrees, evidenced by the four points $a, b, c, d$ in Fig. 8B. The oscillation is coupled to the flexibility of the 634–640 loop connecting the linker to the core domain, a loop that is known to be flexible also in the native enzyme, because it is not detected in the x-ray diffraction structure of the non-covalent protein DNA-complex (6).

**DISCUSSION**

The results presented here demonstrate that the hTop1A653Pp characterized by a single mutation on the linker domain is active in vivo and displays a striking CPT resistance (Fig. 1, panels A and B). The occurrence of CPT resistance because of a mutation far from the drug binding site is an intriguing finding, because it cannot be simply explained by a structural alteration of the protein with a consequent decreased affinity of the enzyme-DNA complex for CPT. The drug is able to bind the hTop1A653Pp as shown by its perturbation of the level of DNA cleavage displayed in Fig. 2. The basis for the reduced sensitivity of the mutant to CPT must therefore be found in some step beyond the binding of the drug to the enzyme-DNA covalent complex. It is interesting to notice that similar conclusions were reached in a previous work by Stewart et al. (12), comparing the properties of the hTop1p and the topo58/12 and topo58/6.3 enzymes, two reconstituted proteins, in which the linker domain is, respectively, either not bonding to the core domain or completely deleted (12). In that work it was shown that detachment of the linker from the core domain makes the enzyme less sensitive to CPT. Here we show that a single mutation is enough to induce CPT resistance, further confirming the importance of a functional linker for the proper response to the drug.

In this context it is interesting to analyze in more detail the functional properties of the mutant in comparison to the wild type enzyme. A characteristic of the mutant is that the cleavage-religation equilibrium ($K_{cr} = k_r/k_c$) is strongly shifted toward religation (see Fig. 2). Such a shift could be because of either an increase of the rate of religation $k_r$ or a decrease of the rate of cleavage $k_c$. The results indicate that the variation in the cleavage-religation equilibrium is because of a 3-fold increase of $k_r$ (see Figs. 3 and 4) and almost a 2-fold decrease of $k_c$ (Fig. 5, panels A and B) for the hTop1A653Pp in the absence of CPT compared with the wild type protein. The addition of CPT dramatically reduces the religation rate for the wild type protein but does not show any effect on the hTop1A653Pp (see Figs. 3 and 4).

The MD results indicate that the main effect of the single mutation is an increased flexibility that brings the linker to sample a larger conformation space, as shown by the root mean square fluctuation data in Fig. 7. The conformations visited during the simulations by the wild type enzyme maintain a dihedral angle close to the starting values of 52 degrees, whereas the mutant progressively change its orientation until a final dihedral value of 26 degrees, as shown in Fig. 8B. This high flexibility is not coupled to a loss of the secondary structure, which is well conserved over the whole trajectory (Fig. 8). The linker domain is known to be a relatively flexible region also in the native state as shown by the different conformations found in the crystals of the DNA-topoisomerase complex (12, 31) and as confirmed by the recent structure of the binary complex.
Linker Flexibility and Camptothecin Sensitivity of hTop1p

Figure 8. MD conformations visited during the simulation. Panel A, superposition of 100 MD structures, taken at 10-ps intervals during the dynamics of wild type and A653P linker domain. Panel B, the starting and final MD structures were superimposed to the x-ray DNA-Top1 complex structure (Protein Data Bank entry 1A36). Wild type and A653P starting structure is represented in green. Wild type and A653P final structures are represented in blue and red, respectively. The starting dihedral angle abcd (52 degrees), the final dihedral angle for the wild type linker domain abcd (52 degrees), and the final dihedral angle for the A653P mutant linker domain abcd (45 degrees), and the final dihedral angle for the A653P mutant linker domain abcd (26 degrees) are also represented.

topo1s-DNA complex compared with the Topotecan ternary complex (8). In this recent work the linker domain was not visible in the electron density map of the non drug-bound protein, but it was detected in the Topotecan-bound complex, indicating a role of drug binding in the linker fluctuations. Evidence of a disordered linker domain has been found also for the topo58/12 reconstituted enzyme, which has catalytic properties similar to those displayed by the topo58/6.3 form that lacks the linker domain (28). The key role of this domain in the controlled rotation step has been also demonstrated for the topo70AL enzyme, having the region 680–688 deleted, resulting in an enzyme with a shortened linker domain (32). The topo70AL enzyme displays, as the single mutant here presented, a slight reduction in activity when compared with the wild type, a cleavage-religation equilibrium shifted toward the religation, and CPT resistance.

Taken together our and the above mentioned results demonstrate that the linker is an essential domain that slows the religation step, permitting the wild type enzyme to remain associated to DNA for a larger number of cleavage and religation rounds. The slowing of the religation step makes the wild type enzyme sensitive to camptothecin, because the drug has the possibility to stabilize the covalent protein-DNA intermediate. On the other hand, a disabled linker, as the one present in the mutant here investigated, produces an enzyme with a reduced DNA binding affinity and shifts the cleavage-religation equilibrium toward religation.

Two MD simulation studies have indicated the occurrence of long range communications between different topoisomerase domains (33, 34). A specific long range communication, involving the drug binding region, the geometry of the active site, and the linker domain has been demonstrated by the differential detection of the linker domain through x-ray diffraction only in the topoisomerase-DNA complex in the presence of Topotecan (8). It is reasonable to think that the linker domain increased flexibility, found in the hTop1A653P, may alter the conformational changes imposed by drug binding, giving rise to a CPT-resistant enzyme. According to the above hypothesis, addition of camptothecin restores the cleavage-religation equilibrium of the hTop1A653Pp to nearby that of wild type enzyme in the absence of CPT (Fig. 2). In this context it must be acknowledged that the existence of a natural occurring CPT-resistant mutant having a disabled linker domain has been hypothesized by Ireton et al. (32), and here for the first time we demonstrate that the single point A653P mutation confers these properties to human topoisomerase I.

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