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Mutation of Gly717Phe in human topoisomerase 1B has an effect on enzymatic function, reactivity to the camptothecin anticancer drug and on the linker domain orientation

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ABSTRACT

Human topoisomerase 1B controls the topological state of supercoiled DNA allowing the progression of fundamental cellular processes. The enzyme, which is the unique molecular target of the natural anticancer compound camptothecin, acts by cleaving one DNA strand and forming a transient protein–DNA covalent adduct. In this work the role of the Gly717 residue, located in a α-helix structure bridging the active site and the linker domain, has been investigated mutating it in Phe. The mutation gives rise to drug resistance *in vivo* as observed through a viability assay of yeast cells. *In vitro* activity assays show that the mutant is characterized by a fast religation rate, only partially reduced by the presence of the drug. Comparative molecular dynamics simulations of the native and mutant proteins indicate that the mutation of Gly717 affects the motion orientation of the linker domain, changing its interaction with the DNA substrate, likely affecting the strand rotation and religation rate. The mutation also causes a slight rearrangement of the active site and of the drug binding site, providing an additional explanation for the lowered effect of camptothecin toward the mutant.

1. Introduction

DNA topoisomerases are key enzymes that modulate the topological state of DNA through the breaking and rejoining of DNA strands. These enzymes have been shown to be essential in all processes of DNA metabolism such as replication, transcription, recombination and chromosomal segregation and are key enzymes as molecular target for anticancer and anti-microbial therapies [1–3]. Due to its importance the human enzyme has been extensively studied during the last decades in order to detect the role of the different domains that compose the protein, both in terms of activity and drug sensitivity [4,5]. Human topoisomerase 1 (hTop1) is a 765 residues protein of 91 kDa that exerts its action by cleaving one strand of the DNA through a tyrosine residue that operates a nucleophilic attack on the strand, supported by four positive residues that altogether form the catalytic pentad (Arg488, Lys532, Arg590, His632 and Tyr723). The protein is composed by an N-terminal domain dispensable for the catalytic activity (residues 1–214), a core domain divided in subdomain I (215–232; 320–433), II (233–319) and III (434–635), a linker domain (636–712) and a C-terminal domain (713–765) [3]. Subdomains I and II form the CAP region, while subdomain III and C-terminal form the CAT region containing the active site, the two regions forming a "clamp" structure that wraps around the DNA substrate, with the linker domain protruding outside of the globular shape of the protein, as shown by the different X-ray structures of the protein solved in non-covalent or covalent complex with the nucleic acid in the presence or the absence of inhibitors [6–10]. HTop1 has been studied since the early 80s, when it was discovered to be inhibited by the natural compound camptothecin (CPT) [11], whose derivatives topotecan and irinotecan have been approved by the FDA for clinical use in anticancer therapy [12]. CPT binds the covalent Top1–DNA complex, slowing down the religation of the cleaved DNA strand. The binding of CPTs to the cleavage complex is *per se* reversible, but the compound becomes lethal due to the collision of the stalled enzyme–DNA complex with the replication fork [5,10,13].

Point mutations that affect the enzyme reactivity to CPT have been deeply analyzed. Most of the mutations that affect CPT sensitivity are located either in the drug binding site or on the linker domain [14–19].

Abbreviations: hTop1B, human topoisomerase 1B; CPT, camptothecin; TPT, topotecan; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; DTT, dithiothreitol; BSA, bovine serine albumin; TBE, tris-borate-EDTA; PMSF, phenylmethanesulfonylfluoride

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The flexibility of the linker can regulate the religation process affecting CPT sensitivity [17], as reported for the Ala653Pro mutant characterized by an enhanced linker mobility and a fast religation that likely doesn't allow the cleavage complex to be enough persistent to permit the interaction with the drug. In agreement, the double Asp677Gly/Val703Ile mutant, hypersensitive to CPT shows a low religation rate coupled to reduced linker mobility [18]. However it has been shown that CPT resistance is not only correlated to enhanced linker mobility but, as demonstrated for Thr729Lys/Pro, Glu710Gly and Arg634Ala mutants, also to a perturbation of motion correlation between the linker and the C-terminal domain, containing the active site tyrosine [20–22]. These data indicate that the linker domain and the active site of the protein, even if far one from the other, are able to communicate so that a mutation on the linker may have an effect on the active site. The communication goes in both directions since a mutation of a residue close to the active site is also able to perturb the linker flexibility as observed in the Thr718Ala mutant [23].

The importance of the linker has been underlined also in the yeast enzyme that has a linker longer than the human one. Van der Merwe and Bjornsti have shown that mutation of Gly721Phe in yeast Top1 gives rise to a CPT resistant mutant and they hypothesized that this could be due to enhanced linker flexibility, but they did not go through the analysis of the different steps of the catalytic cycle [24]. Motivated from these results we have now mutated in hTop1 the Gly717 residue, that on the basis of the alignment corresponds to Gly721 of the yeast enzyme and the Gly717Phe mutant has been analyzed through a coupled functional and computational analysis to understand its behavior at molecular level. We have specifically selected the Gly717Phe mutation since we hypothesized that introduction of a large lateral chain could strongly perturb the structural dynamical–functional properties of the enzyme. We confirm that the mutation confers CPT resistance, underlining the similarity between the yeast and the human enzyme, and we demonstrate that the mutation induces a fast religation rate likely correlated to a different orientation of the linker domain and a slight rearrangement of the active site as observed by molecular dynamics simulation.

2. Materials and methods

2.1. Chemicals, yeast strains and plasmids

Dimethyl sulfoxide (DMSO) and camptothecin (CPT) were purchased from Sigma-Aldrich. CPT was dissolved in 99.9% DMSO to a final concentration of 4 mg/mL (11.5 mM) and stored at −20 °C. Oxindolimine copper(II) compound ($[Cu(isapn)]^{2+}$) was dissolved in 99.9% DMSO to a final concentration of 17.7 mM and stored at −20 °C. Anti-FLAG M2 monoclonal affinity gel, FLAG peptide and anti-FLAG M2 monoclonal antibody were provided by Sigma-Aldrich. *Saccharomyces cerevisiae* Top1 null strain EKY3 (ura3-52, his3Δ200, leu2Δ1, trp1Δ63, top1::TRP1, MATα) was used to express the hTop1 gene. Single copy plasmid YCpGAL1-e-hTop1, in which the hTop1 is expressed under the galactose inducible promoter, was described previously [25]. The hTop1Gly717Phe was generated by oligonucleotide-directed mutagenesis of the YCpGAL1-hTop1. The epitope-tagged construct YCpGAL1-e-hTop1 contains the N-terminal sequence FLAG: DYKDDDDY (indicated with 'e'), recognized by the M2 monoclonal antibody. The epitope-tag was subcloned into YCpGAL1-hTop1Gly717Phe to produce the YCpGAL1-e-hTop1Gly717Phe.

2.2. Drug sensitivity assay

Yeast EKY3 strains were transformed with YCp50, YCpGAL1-e-hTop1 and YCpGAL1-e-hTop1Gly717Phe by LiOAc treatment and selected on synthetic complete (SC)-uracil medium supplemented with 2% dextrose. Cultures of transformants were grown to an Abs595 of 0.3 and 5 μL aliquots of serial 10-fold dilutions were spotted onto SC-uracil plates plus 2% dextrose or 2% galactose, with or without the indicated concentrations of CPT. Plates were incubated at 30 °C for 3 days.

2.3. Protein purification

The transformed EKY3 yeast cells with YCpGAL1-e-hTop1 and YCpGAL1-e-hTop1Gly717Phe were grown to an Abs595 of 1.0 on SC-uracil plus 2% dextrose. Then they were diluted 1:100 in SC-uracil plus 2% raffinose and grown overnight until Abs595 of 1.0 again. After 6 h induction with 2% galactose, the cells were then centrifuged, washed with cold water and resuspended in 2 mL TEEG buffer per gram wet cells [50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10% glycerol and protease inhibitors cocktail from Roche, supplemented with 0.1 mg/mL sodium bisulfate, 0.8 mg/mL sodium fluoride, 1 mM PMSF and 1 mM DTT]. The 0.5 volumes of 425–600 μm diameter glass beads were mixed with cells, the cells were disrupted by vortexing 30 times for 30 s alternating with 30 s on ice and then centrifuged for 30 min at 15,000 *g* to collect supernatant. To purify the interested protein, an anti-FLAG M2 monoclonal affinity gel (Sigma-Aldrich) was equilibrated to the columns as described in the technical protocol and washed with 20 volumes of TBS (50 mM Tris–HCl pH 7.4 and 150 mM KCl) supplemented with the protease inhibitors. Then the whole extracts were applied to the columns. 1 mg of FLAG peptide (DYKDDDDK), diluted to five column volumes of TBS buffer, was adopted to elute e-hTop1 or e-hTop1Gly717Phe protein by competition binding sites. Fractions of 500 μL were collected and stored in 40% glycerol at −20 °C. Protein levels and integrity were assessed by immunoblot with the monoclonal anti-FLAG M2 antibody as described in D'Annessa et al (2013) [20].

2.4. DNA relaxation assays

HTop1 activity was assayed with a DNA relaxation assay. Equal amount of hTop1 or hTop1Gly717Phe protein was incubated with 0.5 μg of negatively supercoiled pBlue-Script KSII(+) DNA, present in both dimeric and monomeric forms, in 30 μL of reaction buffer containing 20 mM Tris–HCl pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 5 μg/mL acetylated bovine serum albumin and 150 mM KCl, in the absence and presence of CPT or oxindolimine copper(II) compound ($[Cu(isapn)]^{2+}$) recently tested as a hTop1 inhibitor [26]. Reactions were incubated at 37 °C and terminated with 0.5% SDS at each indicated time-course point. The samples were resolved at a 1% (*w*/*v*) agarose gel in running buffer containing 48 mM Tris, 45.5 mM boric acid, and 1 mM EDTA at 10 V/cm. After staining with ethidium bromide (0.5 μg/mL) and washing with water, the gels were first exposed to UV light for 30 min to induce photo-nicking of the plasmid, then stained again with ethidium bromide (0.5 μg/mL) for 20 min and photographed using a UV transilluminator [17]. The remaining supercoiled plasmid percentage, quantified using ImageJ software (http://rsbweb.nih.gov/ij/) was normalized to total amount of DNA in each lane and plotted as a function of time.

2.5. Cleavage kinetics

Oligonucleotide CL14-U (5′-GAAAAAAGACTUAG-3′) was radiolabelled with [γ 32P] ATP at its 5′-end [27]. The CP25 complementary strand (5′-TAAAAATTTTTCTAAGTCTTTTTTC-3′) was phosphorylated at its 5′-end with unlabeled ATP. The CL14-U strand was annealed with a 2-fold molar excess of CP25 to obtain the CL14-U/CP25 suicide substrate, which contains an hTop1 preferred high affinity cleavage site. Further, equal amount of hTop1 or hTop1Gly717Phe enzyme was incubated with 20 nM suicide substrate in 20 mM Tris–HCl pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 5 μg/mL acetylated BSA, and 150 mM KCl, in absence or presence of 50 μM $[Cu(isapn)]^{2+}$ [26] at 25 °C. Five microlitre aliquots were removed at indicated time points and the reaction was stopped with 0.5% (*w*/*v*) SDS. The samples were directly analyzed by denaturing 7 M urea/20% polyacrylamide gel electrophoresis in running buffer containing 48 mM Tris, 45.5 mM boric acid, and 1 mM EDTA.