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Analysis of the active site mechanism of Tyrosyl-DNA phosphodiesterase I: a member of the phospholipase D superfamily

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Abstract

Tyrosyl DNA phosphodiesterase I (Tdp1) is a member of the phospholipase D superfamily and hydrolyzes 3'phospho-DNA adducts via two conserved catalytic histidines, one acting as the lead nucleophile and the second as a general acid/base. Substitution of the second histidine specifically to arginine contributes to the neurodegenerative disease SCAN1. We investigated the catalytic role of this histidine in the yeast protein (His432) using a combination of X-ray crystallography, biochemistry, yeast genetics and theoretical chemistry. The structures of wild type Tdp1 and His432Arg both show a phosphorylated form of the nucleophilic histidine that is not observed in the structure of His432Asn. The phosphohistidine is stabilized in the His432Arg structure by the guanidinium group that also restricts access of a nucleophilic water molecule to the Tdp1-DNA intermediate. Biochemical analyses confirm that His432Arg forms an observable and unique

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SG, EQC, NJ, DB, NB and RCAMVW did the experiments. SWW and RCAMVW designed experiments and supervised the study. SG, EQC, DB, SWW and RCAMVW wrote the manuscript.

Accession numbers

Coordinates and Structure factors were deposited in the protein database (<http://www.rcsb.org/>) under accession numbers (PDB ID): 3SQ3 (H182A); 3SQ5 (H432N); 3SQ7 (H432N_Glu) and 3SQ8 (H432R).

Conflict of interest

The authors declare that they have no conflict of interest.

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Tdp1-DNA adduct during catalysis. Substitution of His432 by Lys does not affect catalytic activity or yeast phenotype, but substitution with Asn, Gln, Leu, Ala, Ser and Thr all result in severely compromised enzymes and Top1-camptothecin dependent lethality. Surprisingly, His432Asn did not show a stable covalent Tdp1-DNA intermediate which suggests another catalytic defect. Theoretical calculations revealed that the defect resides in the nucleophilic histidine and that the pKa of this histidine is crucially dependent upon the second histidine and the incoming phosphate of the substrate. This represents a unique example of substrate-activated catalysis that applies to the entire phospholipase D superfamily.

Keywords

Biochemistry; DNA repair; Enzyme mechanism; X-ray crystallography; yeast genetics

Introduction

Eukaryotic cells carry a variety of DNA repair proteins to quickly deal with DNA damage and to ensure the faithful processing of their genome. Tyrosyl-DNA phosphodiesterase I (Tdp1) is a highly conserved eukaryotic DNA repair enzyme that removes DNA-adducts within single-strand breaks (SSBs) and double-strand breaks (DSBs). Tdp1 was originally identified by its ability to hydrolyze a 3'phosphotyrosyl linkage that is transiently formed between the eukaryotic DNA topoisomerase I (Top1) active site tyrosine and the 3'-phosphoryl end of one strand of DNA during the DNA unwinding process^{1; 2}. Such covalent adducts can be converted into persistent SSBs or DSBs if the normally reversible Top1 catalyzed reaction is stalled, for example by camptothecins (CPTs) or DNA damage, to create so-called 'cleavage complexes'^{3; 4; 5; 6; 7; 8; 9}. Subsequent studies have extended the Tdp1 substrate spectrum to include different 3'phospho-DNA adducts, such as 3'phosphoglycolate moieties induced by endogenous reactive oxygen species, ionizing radiation, or the radiomimetic drug bleomycin^{9; 10; 11}. Tdp1 is now regarded as a general 3' DNA end-processing enzyme that acts within the SSB repair complex to remove adducts and to prepare the broken DNA strand for religation¹².

Tdp1 is a member of the phospholipase D (PLD) superfamily which contains paired catalytic histidine and lysine residues within two conserved HxK(x)₄D motifs¹³. Although, Tdp1 shares the conserved His and Lys residues in these two motifs, the Asp residue is not present which places Tdp1 in a novel subclass (HxK-motif) within the PLD superfamily¹³. Mechanistic and structural studies on human Tdp1 (hTdp1)^{11; 13; 14; 15; 16; 17; 18} and yeast (*Saccharomyces cerevisiae*) Tdp1 (yTdp1)^{9; 19} have revealed that the conserved histidine residue in the first motif or the 'N-terminal histidine' (His263 in hTdp1 and His182 in yTdp1) functions as the lead nucleophile to cleave the 3' adduct from the DNA. The 'C-terminal histidine' within the second motif (His493 in hTdp1 and His432 in yTdp1) functions as a general acid/base to first protonate the leaving adduct and then activate a water molecule for the second nucleophilic attack that resolves the Tdp1-DNA covalent enzyme intermediate (Fig. 1). Upon dissociation, Tdp1 leaves a SSB, and the 5' and 3' ends undergo further processing prior to religation by DNA ligase.

Substitution of the C-terminal histidine by an arginine in hTdp1 (H493R) has been identified as a molecular lesion in patients with the hereditary neurodegenerative disease spinocerebellar ataxia with axonal neuropathy or SCAN1²⁰. Recent *in vitro* studies have shown that H493R is impaired in resolving the Tdp1-DNA covalent intermediate¹⁶. This suggests that the SCAN1 phenotype could be related to this defect in catalytic mechanism, but this has not been shown *in vivo/cell*. The Tdp1 knockout mice does not reveal any of the SCAN1 symptoms as would be predicted if the enzyme was simply inactive^{21; 22; 23}, but

introduction of the His to Arg or the “SCAN1” knock-in mouse has not been reported. However, the analogous yeast mutant enzyme (H432R) shows a similar CPT/bleomycin phenotype¹⁹.

To more fully understand the catalytic role of the C-terminal histidine, and the implications of substituting this residue in terms of Tdp1 catalysis, we describe a comprehensive structure-function analysis of the yeast enzyme active site with particular emphasis on the second HxK motif.

Results

Increased expression of Top1 phenocopies cells expressing low levels of Top1 incubated with CPT

We previously reported that the yeast H432R mutant protein induces enhanced sensitivity to both CPT and bleomycin, and that the H432N mutant results in a Top1-dependent, but CPT-independent, self-poisoning phenotype¹⁹. In these experiments, we reasoned that increased expression of Top1 generates Top1-DNA levels similar to those in yeast strains expressing low Top1 levels treated with sub-lethal CPT concentrations. To confirm this, we expressed *TOP1* from an ARS/CEN plasmid (1 to 4 copies per cell) using either the strong constitutive *GPD* promoter for high levels of Top1¹⁹ or its own *TOP1* promoter for low levels of Top1, while *TDP1* was expressed from the *GAL1* promoter of a second ARS/CEN plasmid. As anticipated, elevated levels of Top1 without CPT display the same phenotype as lower levels of Top1 treated with a sub-lethal (5 µg/ml) concentration of CPT (Fig. 2a panel ↑Top1 and Top1/CPT, respectively). In addition, we quantified the clonogenic survival of the ↑Top1-Tdp1 co-transformants relative to vector control, and demonstrated a 4-log drop in cell viability induced by Tdp1H432N expression in a ↑Top1 background (Fig. 2a). Surprisingly, overexpression of wild type yTdp1 and Top1 shows a slight toxicity and a slow growth phenotype compared to the Tdp1 delete (Fig. 2a)¹⁹. This could be the result of the single strand nick that every Tdp1 protein leaves behind when it dissociates from the DNA, even when Tdp1 is able to dissociate faster than Top1 from the DNA. The ends within this nick contain the chemical groups (3'-phosphate and 5'-hydroxyl) which need to be reversed before DNA ligase can religate the DNA strand. We propose that the increased number of single strand nicks results in the observed slow growth and the slight decrease in cell viability. In the Tdp1 delete strain, there is no increase in single strand nicks or possible sequestering of DNA repair proteins by Tdp1 or its actions, and Top1 will dissociate from the DNA and leave behind an intact strand. To confirm that the Tdp1 mutant phenotypes require an active Top1 enzyme, we co-expressed H432N with the catalytically inactive Top1Y727F mutant^{19; 24} and demonstrated no alterations in cell viability (Fig. 2b). Thus, Top1 overexpression represents a physiologically relevant substitution in cells with low levels of Top1 treated with sub-lethal exposure to CPT. Therefore, phenotypes observed with overexpression of Top1 (↑Top1) will henceforth be more accurately called Top1-CPT dependent.

Crystallographic analysis of active site point mutants

To directly visualize the active sites of yTdp1 mutants H432R and H432N, we purified and crystallized the two proteins and determined their crystal structures. Both mutants generated crystals very similar to those of the wild type protein in space group P1 with four molecules in the asymmetric unit¹⁹, and resulted in high quality structures at 2.1 Å for H432R and 2.3 Å for H432N (Table 1). Compared to the wild type protein (PDB ID: 1Q32)¹⁹, the two mutant structures are practically identical with an overall RMSD (α-carbons) of 0.60 Å and 0.49 Å for H432R and H432N, respectively. The key active site residues in the wild type structure (Fig. 3a) superimpose almost perfectly on their counterparts in the two mutant

structures (Fig. 3b, c). These structures suggest that the phenotypes of the two mutants are related to active site chemistry rather than to simple distortion of the substrate binding pockets.

A clue as to the differing chemistries is the presence of strong electron density on the N ϵ 2 nitrogen of His182 in the wild type and H432R structures (Fig. 3a, b) and its complete absence in H432N (Fig. 3c). We interpreted the density, and successfully refined it, as a phosphohistidine that we suggest is generated by Tdp1 acting as a phospholipase on a phosphorylated substrate during expression in *Escherichia coli* (*E. coli*). A similar phosphohistidine that persists through purification and crystallization has been observed in the structure of a bacterial phospholipase D²⁵. In that study, it was proposed that an activated water molecule is not a sufficiently strong nucleophile to remove the phosphate group. We originally misinterpreted this density as water molecules¹⁹ and have now corrected the PDB entry (PDB ID: 1Q32). Refinement suggests that the phosphohistidine is present at ~80% occupancy in the H432R structure and ~60% in the wild type structure where it is less well defined (Fig. 3d). In H432R, the Arg432 guanidinium moiety forms a salt bridge with the covalently bound phosphate, and this may explain the higher occupancy and the superior electron density in all four proteins in the P1 asymmetric unit (Fig. 3b). The complete absence of the phosphate group in H432N suggests among other possibilities, that the putative *E. coli* substrate cannot enter the mutated catalytic pocket and/or that His182 is unable to perform the initial nucleophilic attack in the context of this mutation.

Initial structural studies on H432N showed that the negatively charged glutamate residue directly adjacent to the C-terminal 6xHis affinity tag penetrated the active site of a neighboring molecule in the crystal lattice (Fig. 3f). This additional crystal packing interaction distorted the position of His182, and we subsequently replaced the glutamic acid residue with an additional histidine residue to create a 7xHis tag. Nevertheless, we completed this first structure to 2.0 Å, which we call H432N_Glu (Table 1). The result of the invading glutamic acid is that the side chain of His182 rotated by ~85° (Fig. 3f), and this revealed that the active site is not as rigidly fixed as first thought. To determine whether the mobile His182 has any particular role in organizing the active site locale, we determined the structure of H182A to 2.5 Å (Table 1). Although there are minor adjustments in some of the active site residues (Lys184, Lys434 and Asn465), none are significant and the overall RMSD compared to the wild type structure is 0.72 Å (Fig. 3e).

To visualize the differing gross physical features between the active sites of hTdp1 (PDB ID: 1NOP)¹⁵ and yTdp1, and between the active sites of wild type yTdp1 and the three key mutants H432R, H432N and H182A, we generated accessible surfaces and colored them according to charge (Fig. 4, top panels) and depth (Fig. 4, bottom panels). The hTdp1 and yTdp1 active site clefts have very similar charge characteristics consistent with their conserved residues, but the yTdp1 cleft is more open and less restricted than the hTdp1 cleft. This may explain why the hTdp1 structure does not contain a phosphorylated active site histidine like the yTdp1 structure even though hTdp1 was also expressed in *E. coli*¹³. Compared to wild type yTdp1, H432R shows the clearest differences amongst the mutants; it is shallower due to the introduction of the more bulky side chain, and more electropositive due to the introduction of the positively charged guanidinium group. In contrast, the H432N mutant shows a deeper cleft with a more electronegative charge than wild type and H432R as a result of the Asn side chain character.

Modeling the bound Tyrosyl-DNA substrate

No structure of yTdp1 bound to substrate is available, but such structures have been reported for the human enzyme¹⁵. It has been established that hTdp1 can process not only 3'-pY peptide adducts but also far simpler substrates such as 3'-*p*-methylphenylphosphate ester

DNA (DNA-pMP)¹⁷. We therefore prepared a simplified energy-minimized model of a catalytically competent Michaelis complex by modeling into the wild type yTdp1 structure a three-base DNA-pMP substrate based on substrate complexes of hTdp1 using a procedure that kept all heavy atoms of Tdp1 fixed. In the resulting structural model, the paired active site histidine side chains are perfectly positioned to perform their known functions. The N ϵ 2 nitrogen of His182 is aligned to attack the phosphorous atom from the side opposite the leaving group, and the N ϵ 2 nitrogen of His432 is appropriately oriented to protonate the leaving group. We also prepared models of DNA-pMP complexed with the H432N and H432R mutant structures reported here. The former could be modeled with no need to alter protein atom positions to avoid steric clashes, while the latter required only small adjustments of the Arg432 side chain to accommodate the substrate.

Using these structures, we calculated the pKa values of the key active site residues and compared them with the calculated values in the absence of substrate. All His, Asp, Glu and Cys residues within 15 Å of the active site His182 side chain were considered as sites whose ionization states could vary, and this captured His182, Glu208, His432 and Glu482 (Fig. 5a), as well as His181 and Cys308. Lys, Arg and Tyr residues were considered as fixed in the protonated state, and therefore exerted their influence as part of the “background charge”. The tautomeric states of the His residues (δ or ϵ) were assigned to be δ based on their H-bonding, but His432 and His182 are ambiguous, and calculations were performed with both tautomers as necessary. Modeling and calculations were also performed for the H432N and H432R mutants, and for the wild-type enzyme with the covalently bound phosphate on His182 as observed in the crystal structure.

Calculations of pKa for residues that are buried and/or involved in strong salt bridges often result in extreme pKa values (*e.g.* < 0.0 or > 14.0) because it is electrostatically unfavorable to alter the side chain charges in such environments without compensating structural changes. Such results should not be interpreted as quantitative predictions of pKa measurements but rather as indications that the deprotonated state (in the very low pKa cases) or the protonated state (in the very high pKa cases) is strongly preferred under the conditions in which the structure was determined. They also predict that any change in ionization state would be accompanied by a significant conformational change²⁶. In all of the models, including the apo forms and the Michaelis complexes, the calculated pKa values were <1.0 for His181 and Glu482, and < 0.0 for Glu208 except for the H432N Michaelis complex model where the value was 3.9. This indicates that the deprotonated form of these residues is preferred in all cases. For Cys308, the calculated pKa was > 16 for all cases indicating that the protonated state is preferred. The results for the catalytic histidine residues, 182 and 432, as well as for His394, are more variable. The values are listed in Table 2, and the preferred protonation states and tautomers of the active site His residues based on these calculations is illustrated in Fig. 5b. For the wild-type apo protein, the calculations predict that the deprotonated states of both His182 and His432 are strongly preferred and that they adopt the δ tautomers. These low pKa values are largely the result of the positive potential from the surrounding protein, including nearby lysine and arginine residues.

These predicted ionization states are not fully consistent with their catalytic roles; His182 is appropriately deprotonated with the N ϵ 2 lone pair free to act as a nucleophile. In contrast, His432 is not in the positively-charged state required of a general acid and is predicted to be deprotonated with H δ 1 making a hydrogen bond with Gln214 and an N ϵ 2 lone pair facing the DNA-binding groove. However, a significant change occurs in the substrate-bound wild type model in which His182 remains deprotonated but His432 is now predicted to be protonated. Thus, only upon substrate binding do the protonation states of the two histidine side chains become fully consistent with their catalytic roles: His182 acting as the lead

nucleophile and His432 protonating the leaving phenylate moiety. Analysis of the energy terms in the calculations shows that the negative charge of the bound phosphate group makes the main contribution to the upward shift of pKa values, and that it does so for both histidine residues. A state in which both histidines are protonated is prevented by the repulsion between the two positively charged imidazolium groups that such a state would entail, and substrate binding preferentially protonates His432 to leave His182 free to make the initial nucleophilic attack.

For the mutants H432N and H432R, calculations on the apo state also predicted that His182 would be deprotonated and in the δ tautomer, but they behaved very differently in the context of the Michaelis-complex. In H432R, His182 remains deprotonated, whereas in H432N, His182 becomes protonated. As a result, His182 of the H432R mutant is predicted to be capable of acting as a nucleophile, while in H432N it is not. The exact value of the predicted pKa of His182 in the H432N mutant is sensitive to the details of modeling, but all models consistently agree that the protonated state is strongly preferred near neutral pH. This important result might have been anticipated from the wild-type results where it is only the positive charge of the protonated His432 that prevents His182 from becoming protonated upon binding of the DNA-pMP substrate. In the H432R mutant, the positive charge at position 432 is still present, while in H432N it is not.

Calculations were also carried out for the wild-type protein with His182 phosphorylated, as seen in the crystal structure (Fig. 3a). This phosphorylation is predicted to increase the pKa of His432 to 5.8, but this is not as large an upward shift as is predicted to occur upon binding the DNA substrate, perhaps because the latter carries more negative charge. The calculation thus predicts that, in the (presumably inactive) His182 phosphorylated form, the deprotonated state of His432 would predominate at neutral pH.

His394 is within the broad DNA-binding cleft, but is more solvent-exposed than the catalytic histidines and is not involved in salt bridges. In the model with DNA bound, His394 is closer to the DNA bases than to the backbone phosphate groups. One would therefore expect this residue to have a more typical histidine pKa, and this is indeed the case with the calculated values for most models between 6 and 7.3. The largest shift is in the H432R mutant where its calculated pKa is 4.7, and this can be explained by its proximity to the introduced arginine side chain.

Mutational analysis of His432

The phenotypes of H432R and H432N (Fig. 2)¹⁹, together with the structural results and the modeling described above, suggest that the size and/or chemistry of the side chain at this position dictate catalytic activity. To evaluate a broader range of point mutations, we investigated the following additional yTdp1 mutants; H432K, H432Q, H432L, H432A, H432S and H432T.

We first hypothesized that H432K would induce a similar but less toxic phenotype than is observed for H432R. Similar to histidine, but unlike arginine, lysine often functions as a general acid/base in enzyme catalysis, but the basic side chain could still stabilize the 3'-phosphohistidine covalent intermediate. Indeed, H432K induced a phenotype similar to wild type Tdp1 (Fig. 6a *tdp1* Δ). In contrast, H432Q would be predicted to produce a similar "self"-poisoning phenotype as observed for H432N because the two side chains have similar chemistries and differ by only one carbon length. As expected, H432Q showed a Top1-CPT dependent lethal phenotype equivalent to that of H432N. The outcome of substituting His432 with the hydrophobic residues leucine or alanine, or with the small polar residues serine and threonine was less predictable, yet all four resulted in a Top1-CPT dependent lethal phenotype (Fig. 6a *tdp1* Δ). yTdp1 mutant proteins were stably expressed suggesting

that the Top1-CPT dependent lethal phenotypes were not simply a consequence of mutant protein overexpression (Fig. 6b). Furthermore, as we previously reported for H432N¹⁹, these lethal phenotypes were largely recessive to wild type yTdp1 (Fig. 6a, Tdp1).

In vitro assays of yTdp1 catalytic mutants

The observed variation in yTdp1 mutant phenotypes suggests differences in catalytic activities and/or substrate interactions. To investigate these properties, we employed three different, yet complementary, *in vitro* analyses of the products of a single reaction in which purified wild type yTdp1 or H432R, H432N or H432K mutant proteins were incubated with a 5' end-labeled ³²P-oligonucleotide, which contains a 3'phosphotyrosine modification (3'-pY). The reactions were then divided in three and analyzed for 1) the conversion of the 3'phosphotyrosyl linkage to a 3'phosphoryl-oligonucleotide in denaturing polyacrylamide gel electrophoresis (PAGE); 2) a mobility shift in non-denaturing PAGE to detect covalent and non-covalent protein-substrate interactions, and 3) covalent protein-substrate intermediates by SDS-PAGE. In combination, these assays provided insights into the interactions between the substrate and protein, the overall catalytic rate, and the formation and stability of Tdp1 protein-DNA intermediates.

We and others have shown that the non-catalytic N-terminal domain of Tdp1 is readily removed by proteolysis, but that the deletion does not affect *in vitro* activity to any measurable extent^{13; 17; 19}. Also, Tdp1 undergoes post-translational phosphorylation which could influence its stability and/or activity^{27; 28}. We therefore isolated N-terminal Flag-tagged proteins directly from yeast *tdp1Δ, top1Δ* cells by affinity chromatography. We verified that the Flag-tag did not influence the phenotypes of wild type or mutant yTdp1. Immunoblot analysis revealed that the final purified yTdp1 protein contained both full length (~64 KDa) and an N-terminal deleted yTdp1 (NΔTdp1; ~54 KDa). A similar pattern of Tdp1 protein distribution (Tdp1 and NΔTdp1) was observed in total cell extract (Fig. 6b). Thus, the N-terminus of Tdp1 is also sensitive to proteolysis in eukaryotic cells. To exclude the possibility that the 3'-pY substrate is processed by co-purified contaminants, extracts of galactose-induced control cells were subjected to a mock purification. The "mock" fractions did not show any conversion of the 3'-pY substrate under our reaction conditions.

Relative to wild type yTdp1, where 0.5 fmol was sufficient to convert 75% of the substrate to product, H432K was ~12-fold less active, H432R was more impaired with ~115-fold less activity and H432N was severely impaired with ~560-fold less activity (Fig. 7a, b). A surprising observation is that we could not detect a protein-substrate complex with H432N in either the band shift or the SDS-PAGE assay (Fig. 7c, d) even though we previously reported that H432N and Top1 were in complex with the DNA in yeast¹⁹. This discrepancy may be a consequence of the nature of the substrate and/or the absence of Top1 interactions. In contrast, H432R and H432K both formed protein-substrate complexes in the band shift assay with comparable shifts ranging from 100% to 0% depending on protein concentration (Fig. 7c). When analyzed by SDS-PAGE, all of the H432K-DNA complexes were found to be non-covalent, consistent with the complete conversion of the 3'phosphotyrosyl substrate to 3'phosphoryl product (Fig. 7a). On the other hand, H432R did exhibit the formation of a covalent protein-DNA intermediate that comprised ~10% of the total oligonucleotide at the highest protein concentration (Fig. 7d).

Kinetic analyses of wild type yTdp1 and mutant H432R

To determine the Michaelis-Menten constants of yTdp1 and H432R, we chose to use the highly purified truncated Tdp1 proteins from which we resolved the crystal structure rather than the mix of full length and truncated proteins isolated from yeast. We determined the kinetics at protein concentrations that convert less than 50% of the 3'-pY substrate as

resolved by denaturing PAGE, and kinetic constants were derived from a non-linear regression fit of the data (Fig. 8; Table 3). Wild type yTdp1 (Fig. 8a) displays a second order rate constant $k_{cat}/K_m \sim 1 \times 10^8 \text{ (M}^{-1} \text{ s}^{-1}\text{)}$, similar to that obtained for the human enzyme using similar substrates^{17; 18; 29}. H432R (Fig. 8b) showed approximately 120-fold less activity judged by the k_{cat} values, which is consistent with our estimate of the relative activities based on single turnover experiments using the purified enzymes from yeast (Fig. 7a, b). These data suggest that the phosphohistidine linkage observed at the nucleophilic His182 in the crystal structures of wild type yTdp1 and H432R does not interfere with *in vitro* activity. As noted above, and previously observed for hTdp1, there is clear evidence that H432R generates a DNA covalent intermediate during catalysis which is observed as a trapped species in the wells of the gel. If we consider this intermediate as a stable pseudo product of H432R that can be analyzed by Michaelis-Menten kinetics, the k_{cat} is approximately 2000-fold less than wild type but the K_m is almost identical to the wild-type enzyme (Fig. 8c, Table 3). Thus, under these conditions, initial substrate binding is apparently unaffected in the H432R mutant, while the first nucleophilic attack is delayed.

To analyze the stability of this covalent intermediate over time, we first determined that an enzyme concentration of 0.5 $\mu\text{g}/\mu\text{l}$ would be suitable to view the substrate, the product and the intermediate in a time course experiment (Fig. 8d). At this fixed enzyme concentration, the majority (85%) of the substrate is processed within 5 seconds and converted into either intermediates (~50%) or product (~35%) (Fig. 8e). Within the first minute, all of the substrate is converted and the intermediate concentration is maximal (~60%) and slowly released into product at increasing time points. From these data, we can estimate that the intermediate/product ratio is 1:1 after approximately 3 minutes and that the half-life of the intermediate is approximately 7 minutes. These values are very similar to those obtained for the equivalent hTdp1H493R mutant¹⁶.

The lethal phenotypes of the catalytic mutants are conserved from yeast to human Tdp1

To determine whether the toxic phenotypes induced by H432R and H432N reflect conserved properties of Tdp1, we introduced the equivalent mutations into hTdp1 (H493R and H493N) and expressed the mutant proteins in the *tdp1 Δ top1 Δ* yeast strain. To maintain species-specific interactions, human Top1 was co-expressed with human Tdp1 in these experiments, but the experimental conditions were otherwise identical to those described earlier for the yeast Tdp1 mutants. H493R (the mutation found in SCAN1 patients) induced a low-level increase in CPT sensitivity, while cells expressing H493N exhibited a Top1-CPT dependent lethal phenotype (Fig. 9a). The hTdp1 proteins were stably expressed in yeast cells at similar levels to the yTdp1 proteins suggesting that the observed phenotypes were not simply a consequence of mutant protein overexpression (Fig. 9b). These results demonstrate that hTdp1 functions in yeast in the presence of hTop1, and that the toxic phenotypes of the two yTdp1 mutants H432R and H432N mutants are also manifested by the equivalent hTdp1 mutants. Comparing yeast Top1-Tdp1 to human Top1-Tdp1, it can be seen that the mutant phenotypes are not strictly equivalent, particularly in the case of the His to Asn substitution, but this may be related to species specific differences in hTop1 function in yeast^{30; 31; 32; 33}. It has been shown that hTop1 expression in yeast provides the appropriate DNA topoisomerase I activity and conversely, expression of yTop1 in mammalian cells provides the appropriate activity and CPT sensitivity^{30; 34}. Specifically, expression of hTop1 in yeast results in an increased CPT sensitivity compared to yTop1^{28; 29; 30; 31}, which is not a result of differences in relative protein expression levels of yeast and human Top1 (Fig. 9c). In addition, the N-terminal and linker domains of hTop1 are reciprocal in size and differ in sequence compared to the yeast domains^{30; 31; 32; 33} and show different interactions within a yeast background compared to the equivalent yeast Top1 domains^{31; 33}.

Discussion

The role of Tdp1 in resolving 3'phospho DNA adducts in single-strand break repair has been well characterized, and its importance is reflected in its conservation from yeast to humans^{1; 2; 11; 13; 17; 18; 19}. Tdp1 has evolved by gene duplication from the phospholipase D family of phosphohydrolytic enzymes and has largely retained the twofold symmetrical catalytic center and the catalytic mechanism³⁵. During catalysis, paired histidine residues coordinate a ping pong nucleophilic attack that first releases the 3' adduct to create a phosphohistidine Tdp1-DNA covalent intermediate which is then resolved by an activated water molecule (Fig. 1 and 5a). The N-terminal half of the active site is very similar to the ancestral enzyme, and its histidine (His182 in yTdp1) performs the initial nucleophilic attack to become the phosphohistidine intermediate. The histidine is activated by the acidic residue Glu482 that has been shown by mutagenesis in the human enzyme to be crucial¹⁷, and is present in the ancestral protein³⁵. Our calculations confirm that Glu482 maintains a low pKa and is negatively charged in both the absence and presence of bound substrate.

This report has specifically focused on the role of the C-terminal histidine in the Tdp1 reaction (His432 in yTdp1) and how its mutation modulates catalysis. Our interest in this histidine largely stems from two observations. Its mutation to an arginine residue in human Tdp1 is a molecular lesion found in patients with the rare autosomal recessive neurodegenerative disease spinocerebellar ataxia with axonal neuropathy (SCAN1)²⁰, and its mutation to an asparagine residue generates a toxic enzyme (Fig. 9). In the phospholipase D superfamily, it has been assumed that the C-terminal histidine has two major roles in the catalytic mechanism; it first provides a proton to the leaving group and then activates a nucleophilic water molecule to resolve the covalent intermediate¹⁸. Our studies have shown that the C-terminal histidine has a third role in catalysis which is to become preferentially protonated upon substrate binding leaving the N-terminal histidine deprotonated and free to perform the initial nucleophilic attack. In the C-terminal half of the Tdp1 active site, the "histidine-activating" residue has been modified compared to the ancestral protein by the replacement of the acidic residue by a polar asparagine (Gln214 in yTdp1) (Fig. 5a). Our calculations on yTdp1 reveal that His432 is actually neutral in the absence of substrate, and that it is substrate binding that raises its pKa and leads to the required protonation. This represents a rather unique example of substrate-induced enzyme activation where the negatively charged phosphate of the bound DNA is the activator. In the Michaelis complex, His432 is not directly solvent accessible, but others have shown that the proton probably reaches His432 via a proton relay involving Lys434 and Asn465¹⁷. The calculations also reveal that the N-terminal histidine remains deprotonated in the presence of the negatively charged DNA substrate because the preferential protonation of the C-terminal histidine disfavors the additional protonation of the N-terminal histidine due to the resulting charge repulsion.

The disruption of Tdp1 catalysis by mutations of the C-terminal histidine have previously been explained by the unquestioned impact on the acid/base properties that are required to protonate the leaving group and subsequently activate the resolving water molecule. However, our discovery of the third role of the histidine provides new insights into the catalytic defect that are supported by mutagenesis studies. We have shown that mutation of the C-terminal histidine to either arginine or lysine preserves the ability of Tdp1 to perform the initial nucleophilic attack and release of tyrosine, albeit at reduced efficiency and retention of the DNA within the Tdp1 catalytic pocket, while mutation to other residues does not. Replacement by arginine or lysine would continue to provide the required positive charge that maintains the N-terminal histidine in a nucleophilic state. In contrast, mutations to either neutral or hydrophobic residues would not supply the positive charge and the N-terminal histidine would become protonated upon substrate binding. We verified this by

performing calculations on the H432N mutant, and these showed that DNA binding indeed leads to the uptake of a proton by His182 and an enzyme severely compromised in catalysis. This was confirmed by the *in vitro* activity assays which showed that H432N has 560-fold reduced activity compared to wild type.

Our studies also provide insights into why the paired active site histidine residues operate in the observed order and not in the reverse order such that the DNA is first released to generate an adduct-phosphate-histidine covalent intermediate. Although our calculations clearly show that His182 is poised to act as a nucleophile in the Michaelis complex, and that His432 has the required acid/base characteristics, this is not the whole story. The same order has also been observed in a bacterial phospholipase D enzyme that is also an intramolecular dimer but in which the twofold symmetry has largely been retained including activating acidic residues adjacent to both histidines²⁵. Our energy minimized structure of the Tdp1-substrate complex prior to catalysis shows very clearly that the N-terminal histidine is more appropriately oriented for nucleophilic attack whereas the C-terminal histidine is more appropriately positioned to donate a proton to the leaving group. Thus, substrate binding and orientation may have been the original reason for the observed order in more primitive members of the family, and subsequent mutations in Tdp1 that tailored the enzyme to its unique substrate may have augmented this feature of the catalytic mechanism.

In terms of the resolution phase of catalysis, our mutagenesis data reveal that H423R and H432K are relatively active enzymes and still able to perform this step. The activities of H432R and H432K compared to wild type Tdp1 are only 115- and 12-fold reduced, respectively, and the phenotype of H432K mimics that of wild type Tdp1. H432R is clearly the least active of the pair, and our crystallographic analysis shows that the guanidinium group of Arg432 could sterically block access of a resolving water molecule to the phosphohistidine group of the covalent intermediate and also stabilizes the phosphohistidine by hydrogen-bonding and ionic interactions. Based on this structure, we predicted that H432K should also stabilize the intermediate but be less defective because the lysine side chain has more favorable acid/base characteristics and is less bulky. This prediction was confirmed both *in vivo* and *in vitro*. *In vitro*, both the arginine and lysine mutations increase the lifetime of the non-covalent Tdp1-DNA complex due to the increased positive charge compared to the wild type or the other His432 mutants (Fig. 7c), but this does not affect on cell viability. Only H432R generates a persistent covalent Tdp1-DNA complex (phosphohistidine species), which could affect cell viability. How this feature of the arginine mutation in the human enzyme specifically relates to the SCAN1 phenotype remains to be established, but our studies confirm that the persistent covalent intermediate is a uniquely associated with this mutation.

The reason why the H432N/Q/L/A/S/T substitutions are so toxic to the cell in the presence of Top1-CPT is an intriguing mystery that presents opportunities for more fully understanding the role of Tdp1 in DNA repair. We originally suggested that H432N is even more defective in resolving the covalent intermediate *in vivo*¹⁹, but this is not supported by the current *in vitro* data that fail to demonstrate the covalent intermediate that we observe with H432R. Clearly, it is not the lack of activity of these enzymes that generates toxicity because Tdp1 knockouts are not toxic under the same conditions. An important difference between the *in vitro* experiments and the *in vivo* experiments that needs to be considered in any explanation of toxicity is the size and nature of the substrates. The *in vitro* experiments use minimal substrates while the *in vivo* experiments have much larger substrates and possibly other proteins that form a protein complex bound to genomic DNA. In this scenario, the H432N mutant Tdp1 would form the repair complex in the cell but it would be lingering in the first nucleophilic attack, entrapping both Top1 and Tdp1 in complex on the DNA. As we previously suggested, even if the Tdp1H432N protein is able to perform the

initial nucleophilic attack, the generated phenoxyanion of tyrosine will not be protonated, which would allow the reverse reaction to occur¹⁹. This reformation of the original Top1-DNA covalent complex could then induce either dissociation of the Tdp1H432N protein or induce a ping-pong reaction prolonging the Top1-DNA-Tdp1 complex, which is suggested by our reported observation¹⁹. In contrast, no such complex could form if Tdp1 were simply deleted. Tdp1-Top1 interactions are apparently central to this scenario because replacing the yeast Tdp1 mutant and wild type Top1 with the two equivalent human proteins reproduces the toxic phenotype. This “substrate size-dependent” proposal for toxicity is supported by our previous *in vivo* observations with bleomycin which generates Tdp1 substrates in the cell that more resemble our *in vitro* substrates, namely 3'phosphoglycolate adducts. In this case, H432N is far less toxic to the cell, and a similar observation was reported in cell extracts of Tdp1^{-/-} MEFs supplemented with either human H493R or H493N²³. We also previously noted that 3'phosphoglycolate is less bulky than 3'tyrosyl peptides and could more readily dissociate from or be processed by the less active/functional Tdp1 active site¹⁹.

Finally, the somewhat controversial finding that yeast Tdp1 can also process 5' adducts whereas human Tdp1 cannot was recently resolved by the discovery that TTRAP/Tdp2 performs this function in higher eukaryotes^{1; 36; 37; 38; 39}. The reduced specificity of the yeast enzyme is likely related to the larger and more open active site cleft compared to the human enzyme (Fig. 4). Our finding that His182 has a covalently-bound phosphate group in both the wild type and H432R enzymes may be related to this difference because the phosphate group is not present in the hTdp1 structure¹⁴. Based on previous studies of a bacterial phospholipase D enzyme²⁵, we speculate that the phosphate group originates from a similar *E. coli* phospholipid that is encountered by Tdp1 during expression. The more open active site of yTdp1 may allow processing of this substrate whereas the tighter active site of hTdp1 may not (Fig. 4). As proposed previously²⁵, multiple processing of the phospholipid by Tdp1 can leave a phosphate group bound to the histidine. The phospholipase D active site of Tdp1 has presumably not evolved to efficiently process a phosphohistidine intermediate and an activated water molecule may simply not be a strong enough nucleophile to remove it.

Materials and Methods

Yeast strains, plasmids and drugs

DMSO (dimethyl sulfoxide) and camptothecin were obtained from Sigma (St. Louis). *Saccharomyces cerevisiae* (yeast) strains EKY3 [*top1Δ, TDP1*] (*MATα, ura3-52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::TRP1*) and PFY-68 [*top1Δ, tdp1Δ*] (EKY3, *tdp1Δ::HIS5*) were described in^{39; 40; 41}. Yeast and human Top1 was constitutively expressed at elevated levels from the *GPD* promoter and yTop1 at low levels from its own promoter, in plasmids YCpGDP-TOP1•U, YCpGDP-hTOP1•U or YCpSc-TOP1•U plasmid, respectively^{19; 24; 30}. The yeast Top1Y727F catalytic inactive mutant was expressed from the galactose inducible *GAL1* promoter of the *URA3* plasmid YCpGAL-ytop1Y727F•U^{19; 24}. All of the above strains and plasmids are kindly provided by Dr. Mary-Ann Bjornsti (University of Alabama at Birmingham). For galactose-induced expression of Tdp1, PCR amplified yeast *TDP1*¹⁹ and human *TDP1* cDNA (h*TDP1*), PCR amplified from pCMV6-xl4-hTdp1 (OriGene), were cloned into the YCpGAL1•L (*LEU2*) vector to yield YCpGAL1-Tdp1•L and YCpGAL1-hTdp1•L, respectively. Mutant alleles of yeast and human *TDP1* were generated using a QuikChange Site-Directed Mutagenesis kit (Stratagene). N-terminal Flag-tag versions of *TDP1* alleles were generated by PCR amplification and cloned into pRS426 (*URA3*) vector YEpGAL1•U. Sequences of all *TDP1* alleles were confirmed by DNA sequencing. pRS416 and pRS415 were used control vectors.

Crystallization, data collection and structure determination

Wild-type yeast Tdp1 missing the N-terminal domain (residues 79-539) was previously used to determine the crystal structure¹⁹, and this construct was used to generate point mutants H182A, H432N and H432R from the pET-23b expression vector. The crystallization conditions for each mutant were also very similar; 7-12 mg/ml protein, 17-24% PEG 3350, 100 mM Hepes pH 7.8, 5 mM TCEP, 2-5% hexanediol-1,6 and 200 mM salt (H432N in magnesium sulfate, H432R in magnesium formate and H182A in calcium chloride). Crystals were cryo-protected in 1:1 Paratone N/mineral oil and flash frozen in liquid nitrogen. Diffraction data were collected at the SERCAT beamlines ID-22 and BM-22 at the Advanced Photon Source (Argonne, IL). The structures were determined by molecular replacement using PHASER⁴² and the wild type structure (1Q32) as the search model, and refined using iterative cycles of Refmac with TLS refinement and NCS restraints⁴³. All model building was performed using Coot⁴⁴. Structure figures were created using UCSF Chimera⁴⁵ and Ligplot⁴⁶, electrostatic potentials were calculated with PDB2PQR⁴⁷ and APBS⁴⁸.

Cell viability assays

Semi-quantitative colony formation; Exponential cultures of individual *top1Δ,tdp1Δ* or *top1Δ,TDPI* transformants, co-transformed with the pRS416 (vector control) or indicated yeast TOP1 plasmid and pRS415 (vector control), or YCpGAL1-Tdp1•L vectors, were corrected to OD₅₉₅=0.3, serially ten-fold diluted, and then spotted in 5 µl aliquots onto selective media containing 2% galactose with 0, 1 or 5 µg/ml CPT (as indicated) in a final 0.125% DMSO (Me₂SO) and 25 mM HEPES (pH 7.2).

Human Top1 and human Tdp1 activities were assessed in *top1Δ,tdp1Δ* yeast cells transformed with pRS416 (vector control) or YCpGPD-hTOP1•U and pRS415 (vector control), or YCpGAL1-hTdp1•L vectors. Serially dilutions of cells were spotted onto selective media plates containing 2% galactose with 0 or 0.025 µg/ml CPT in a final 0.00125% DMSO and 25 mM HEPES (pH 7.2). All semi-quantitative cell viability was assessed following incubation at 30°C for 4 days. At least 3 independent experiments were assayed.

Quantitative colony formation; Exponential cultures of *top1Δ,tdp1Δ* cells, transformed with the indicated YCpGPD-TOP1 (↑Top1) and YCpGAL1-Tdp1 vectors, were corrected to OD₅₉₅=0.3, serially ten-fold diluted and 50 µl aliquots were plated onto selective media containing 2% galactose. The number of viable cells that formed colonies were determined after incubation for 4 days at 30°C. Colony formation unit (CFU) was calculated relative to vector control (↑Top1, no Tdp1) and the mean and standard deviation was calculated from at least 3 independent experiments.

Tdp1 protein purification from yeast

N-terminal flag tagged yeast Tdp1 proteins were purified from galactose-induced cultures of *top1Δtdp1Δ* cells, transformed with the appropriate YEpGAL1-flagTdp1•U plasmid. Cell extract in HEEP/2PI (50mM HEPES pH 8.0, 5mM EGTA, 5mM EDTA, 1 mM PMSF and 2x complete Protease Inhibitor Cocktail (Roche)) were added to SP Sepharose fast flow matrix (GE Life Sciences), eluted with HEEP/2PI 0.2-0.3 M NaCl and affinity purified over an anti-flag M2-affinity matrix (Sigma). Flag-tagged proteins were eluted with an excess of 3xflag-peptide (Sigma) in TEEG/p2PI (50mM Tris-HCl pH 7.5, 1mM EGTA, 1mM EDTA, 100 mM KCl, 1% glycerol, 1mM PMSF and 2x complete Protease Inhibitor Cocktail (Roche)). Flag-peptide was removed during concentration with Ultracel-30K (Millipore). Tdp1 protein solution was supplemented with 0.1 µg/µl BSA and 2PI. Protein levels and integrity were determined by immunoblots using anti-flag M2 antibody and rabbit anti-Tdp1

antibody¹⁹, while purity of the samples was determined with Sypro Ruby staining of the 4-12% Bis-Tris SDS-PAGE gel. Sypro Ruby staining of an SDS-PAGE gel revealed >90% of purified protein is Tdp1.

Tdp1 in vitro activity assay

14-mer oligonucleotides were used; the 3'-pY substrate containing a 3' phosphotyrosine (5'-GATCTAAAAGACTT-Y-3') and a product control (3'-PO₄) containing a 3' phosphate (5'-GATCTAAAAGACTT-PO₄-3') (Midland Certified Reagent Company Inc.). These were 5'-³²P end labeled with [γ -³²P]-ATP (Perkin Elmer) and a 3'-phosphatase minus T4 polynucleotidekinase (New England BioLabs). Activity assays used a range of Tdp1 concentrations from ~ 0.05 to 2500 fmol, 250 fmol 5'-³²P-labeled 3'-pY, and reaction buffer (RB: 100 mM KCl, 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 2 mM EGTA and 2 mM DTT), and incubated for 10 minutes at 30°C. The reactions were divided into 3 aliquots, one for a band-shift assay to detect non-covalent/covalent substrate-protein intermediates, the second to measure catalytic activity by conversion of substrate to product, and the third for SDS-PAGE analysis to detect covalent substrate-protein intermediates. All gels were loaded with substrate and product reference (complete reaction assay without enzyme). Aliquot 1 was added to 50% glycerol/0.01% Bromo Phenol Blue buffer, put on ice to stop the reaction and directly resolved on a 20% non-denaturing polyacrylamide (37.5-1; acrylamide-bisacrylamide) gel. Aliquot 2 was added to 2 M urea, heat inactivated for 10 minutes at 72°C, and chilled on ice. 2 μ g of sequence grade trypsin (Promega) was added, incubated over-night at 37°C and the digestion stopped by the addition of USB stop buffer (USB) and incubation for 10 minutes at 90°C. Reactions-products were resolved on a 20% polyacrylamide (19-1; acrylamide-bisacrylamide)/7M urea sequence gel. Aliquot 3 was added to SDS-PAGE sample buffer and heat inactivated for 10 min at 90°C, and the substrate-protein intermediates were resolved on a 4-12% Bis-Tris PAGE (Invitrogen). All assay gels were exposed on PhosphorImager screens for 1 hour or longer, and the screens were scanned with a Storm 865 scanner (GE Life Sciences). Substrate conversion was determined using ImageQuant TL 1D version 7.0 (GE Healthcare) software.

All reactions for kinetic studies of Tdp1 and the SCAN1 mutant were carried out at 30°C in 10 μ l reactions buffered in 50mM NaCl, 25mM Tris-HCl pH 8.5, 100 μ g/ μ l BSA, 2mM EDTA and 2mM DTT. Reactions were stopped with 2X Gel loading buffer (Novex) and denatured (10 minutes at 100°C). Reaction products were resolved on a 10% polyacrylamide/7M urea sequencing gel in 1x TBE. Gels were dried and exposed on PhosphorImager screens up to 12 hours before screens were scanned. Product formation was calculated from the ratio of the integrated intensities of substrate and product bands, imported to Prism 4.0 (Graph Pad) and fitted to the Michaelis Menten equation using non-linear regression. Mean and the standard error of the mean were calculated of at least three independent experiments.

Immunoblotting of yeast cell extracts

Rabbit polyclonal antibodies were raised against Tdp1 residues 356-369 and affinity purified as described in¹⁹ generously provided by Dr. Mary-Ann Bjornsti. The anti-hTdp1 (ab4166) antibodies were purchased from Abcam, while the anti-tubulin antibody (MCA77G) was acquired from AbD Serotec. The anti-flag M2 (F3165) antibody was obtained from Sigma-Adrich. To assay steady state levels of soluble Tdp1 proteins, *top1 Δ ,tdp1 Δ* cells transformed with the indicated YCpGAL1-yTdp1•L, YCpGAL1-hTdp1•L, while for relative levels of N-terminally flag tagged yeast or human Top1 cells were transformed with YCpGAL-flagYTOP1•U or YCpGAL-flaghTOP1•U or vector control. Cells were induced with 2% galactose for 6 hours. Cultures are corrected for lowest OD₅₉₅, cell pellets were washed with ice-cold water and suspended in TEEG/2PI (for Tdp1) or HEEP/2PI (for Top1) and lysed

with acid washed glass beads (Sigma) at 4°C. SDS loading buffer was added to Tdp1 cell extracts and boiled for 10 min. To detect N-terminally flag tagged Top1 cell extracts were partially purified (to remove M2 non-specific reactive protein migrating between 80 and 120 KDa that interfere with the detection of flag-tagged Top1) over the SP Sepharose fast flow matrix. Matrix was washed with HEEP/2PI 0.2 M NaCl and Top1 was eluted with HEEP/2PI 0.7 M NaCl. The collected fractions were concentrated (Ultracel-30K) and SDS loading buffer was added. Samples were boiled for 10 min and were resolved by 4-12% Bis-Tris PAGE in MOPS buffer and blotted to PVDF followed by immunostaining with anti-Tdp1 (yeast or human) or anti-flag (M2) (yeast and human flag-Top1) and anti-tubulin antibodies and visualized by chemiluminescence.

Computational analysis of pKa values of selected active site residues

Coordinates for yeast apo Tdp1 (PDB ID: 1Q32, molecule C)¹⁹ were taken from the RCSB protein database. Addition of hydrogen atoms and assignment of atomic partial charges and radii were done using the LeAP program and the ff03.r1 force field of the Amber11/AmberTools1.4 suite^{49; 50}, and hydrogen positions were energy-minimized while holding heavy atoms fixed. Assignment of His protonation and tautomeric states, and the Oε1 vs Oε2 naming of Glu sidechains (which determines the proton location in the acidic form) in the active site region were done by inspection of hydrogen-bonding geometries. However, for His432 and, in cases where the preferred tautomer of His182 was ambiguous, calculations were done for both tautomers, and the preferred tautomer determined from comparisons of computed pK_a values.

A model of 3'-Tyr-DNA bound to yeast Tdp1 was created by combining the apo structure with 5'-p-dG-dT-dT-3'-pMP (para-methylphenylphosphate) using the structure of the human Tdp1 quaternary complex as a template (PDB ID: 1RFF)⁵¹. Molecular mechanics parameters for the Tyr-DNA adduct were adapted from the standard DNA residues in Amber, and parameters for phosphotyrosine in the -1 charge state contributed by Sticht and co-workers⁵². The modeled DNA coordinates were then energy-minimized in the protein environment while keeping protein heavy atoms fixed. The 3' phosphorous moved from its original position by 0.7 Å, while the overall heavy-atom RMSD between the original and energy-minimized DNA-Tyr was 1.2 Å. Electrostatic calculations for pKa and ionization-state energetics were done with the multiflex and redti programs from the MEAD programming suite^{26; 53}. Partial charges were from Amber ff03 and atomic radii were the Amber H-modified Bondi radii. Partial charges for the phosphohistidine with an unprotonated phosphate group attached to Nε2 were taken from the parameter set contributed by Sticht and co-workers⁵². The interior dielectric constant of the protein was set to 4.0, and the solvent dielectric was 80.0.

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Abbreviations used

SSB single strand break

DSB	double strand break
CPT	camptothecin
SCAN1	spinocerebellar ataxia with axonal neuropathy
PDB	protein data bank
RMSD	root mean square deviation

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Highlights

Resolved crystal structures of Tdp1 H182A, H432R (SCAN1) and H432N catalytic mutants

SCAN1 Arg stabilizes phospho-His linkage, prevents H₂O access & is weak proton donor

Cellular toxicity of H432R & H432N Tdp1 mutants is conserved from yeast to human

Calculations show catalytic His are charged upon binding of phospho-DNA substrate

Catalytic His182 residue shows surprising flexibility in H432N protein structure

Fig. 1.

Tdp1 catalytic mechanism. For simplicity, we only depict the catalytic His residues (yeast residue numbering) and the 3'phosphotyrosyl linkage as substrate. Step 1 can be reversed when the leaving phenoxanion of Tyr is not protonated by the general acid His432, and reforms the original Top1-DNA intermediate. His432 subsequently functions as general base that activates water during step 3, and this results in dissociation of Tdp1 from the DNA, which still contains a single strand nick.

Fig. 2.

Increased expression of Top1 phenocopies cells with low levels of Top1 and treated with CPT. (a) *top1Δtdp1Δ* cells co-transformed with vector control (*top1Δ*), YCpSc-TOP1•U (TOP1) or YCpGPD-TOP1•U (↑Top1) and the indicated YCpGAL1-Tdp1•L or its control vector (*tdp1Δ*), were ten-fold serially diluted and spotted onto selective galactose plates supplemented with or without sub-lethal CPT concentration (5 μg/ml), incubated for 4 days at 30 °C. Transformants of ↑Top1 with indicated Tdp1 vector were used in a quantitative colony formation assay (see Materials and Methods). The mean of 3 independent experiments was calculated relative to vector control and displayed as colony formation units (CFU) with standard deviation (SD). (b) *top1Δtdp1Δ* cells co-transformed with YCpGPD-TOP1•U (↑Top1), YCpGPD-top1Y727F•U (↑Top1Y727F) and the indicated YCpGAL1-TDP1•L or its control vector (*tdp1Δ*), were serially diluted and spotted onto selective galactose plates with or without sub-lethal CPT concentration (1 μg/ml), incubated for 4 days at 30 °C. A representative picture of at least three independent experiments is shown.

Fig. 3.

Crystal structures of the wild type and mutant yeast Tdp1 active sites. (a) Wild type Tdp1, a water molecule that occupies the phenoxyanion binding pocket is shown as a red ball (PDB ID: 1Q32)¹⁹. Electron density for phosphohistidine 182 is contoured at 2σ . Active site residues are labeled. (b) H432R structure. Only the mutation is labeled in red. The dashed purple line represents a salt bridge between the Arg432 guanidinium moiety and the phosphate group (c) H432N. Note the absence of electron density for a covalent phosphate on His182. (d) The electron densities (contoured at 1.5σ) for the four phosphohistidines in the asymmetric unit of wild type Tdp1 (PDB ID: 1Q32). (e) H182A. (f) H432N in structure H432N_Glu (Table 1) where a His-tag glutamate (E541) has penetrated the active site. Note the rotation of His182 in this structure (thin green sticks (no E541 penetration) vs. thick with sticks (with E541 penetration)).

Fig. 4.

Surface representations of the Tdp1 active site cleft. Shown are the active sites of wild type hTdp1 and yTdp1, and the point mutants H432R, H432N and H182A of yTdp1. Top row: surfaces are colored according to the electrostatic potentials calculated at neutral pH from red (negative) to blue (positive). Bottom row: surfaces are colored according to depth from cyan (deep) to maroon (shallow). Golden spheres in the human structure (upper left panel) represent the DNA phosphate-binding pockets (PDB ID: 1NOP)¹⁵. The molecular surfaces were calculated using a 1.8 Å radius Connolly probe. For wild type yTdp1 and the H432R mutant, the covalently bound phosphate group on His182 in the crystal structures was removed for the calculations.

Fig. 5.

Tdp1 catalytic pocket residues. (a) Two dimensional representation of the yeast Tdp1 catalytic environment. Grey and yellow carbons indicate residues from the N-terminal and C-terminal halves of the catalytic pocket, respectively, to highlight the twofold pseudosymmetric active site, using Ligplot⁴⁶. Putative hydrogen bonds (lengths in Å units) are shown as dashed lines. Glu482 and Gln214 are involved in regulating the charged states of the catalytic histidines 182 and 432, respectively. The observed phosphorylation of His182 is shown in orange and a water molecule that occupies the phenoxanion binding pocket of wild type Tdp1 is shown as a blue ball (PDB ID: 1Q32)¹⁹. The binding site of the +1 phosphate of the DNA substrate (derived from PDB ID: 1NOP)¹⁵ is indicated by an orange transparent phosphate group adjacent to Ala470. (b) Predicted protonation states of the Tdp1 active site histidines. The protonation states are based on their computationally determined pKa values as described in the text and presented in Table 2. Mutations are labeled in red font. Top Row: The crystallographically determined apo structures of wild type yTdp1 and mutants H432R and H432N. Bottom Row: The computationally refined models of the three active sites containing the bound model substrate shown as transparent red sticks (see text for details). The green balls represent protons on the histidine Nδ1 and Nε2 nitrogen atoms. Note in the wild type enzyme that the protonation states are only consistent with catalysis when the phosphate group of the substrate is present (bottom left panel). Note also in H432N that His182 is fully protonated and therefore catalytically incompetent when the substrate is bound (bottom right panel).

Fig. 6.

Lys and His at 432 maintain cell viability. (a) Serial dilutions of *top1Δtdp1Δ* (*tdp1Δ*) cells or *top1ΔTDP1* (Tdp1) cells, co-transformed with vector control (*top1Δ*) or YCpGPD-TOP1•U (↑Top1) and the indicated YCpGAL1-Tdp1•L or its control vector (vector), were serially ten-fold diluted and spotted onto selective galactose plates incubated at 30 °C for 4 days. (b) Total cell extracts from *top1Δtdp1Δ* cells co-transformed with vector control (*top1Δ*) and the indicated YCpGAL1-Tdp1•L or its control vector used in (a) were resolved on 4-12% SDS-PAGE and stained with anti-yeast Tdp1 (triangle points to full length Tdp1 protein while circle shows N-terminal truncated Tdp1 protein). blot is subsequently stripped and re-stained with anti-αTubulin. A representative picture of at least three independent experiments is shown.

Fig. 7.

In vitro analysis of Tdp1 enzymatic activity. N-terminally flag-tagged Tdp1 protein ranging from 2500 to 0.5 fmol was incubated with 250 fmol of 5'-³²P labeled 14-mer oligonucleotide with a 3'phosphotyrosine at 30°C for 10 minutes. Reactions were split in three and analyzed as described in (a), (c) and (d) using a phosphoimager. (a) The conversion of the 3'phosphotyrosine to 3'phosphate in a 20% denaturing polyacrylamide gel. (b) The mean and standard deviation of substrate to product conversion (product/[product + substrate]) from (a) were quantitated by phosphoimager analysis of at least three independent experiments. Tdp1 (circle), H432N (triangle), H432R (square) and H432K (diamond); (c) Detection of non-covalent/covalent protein-oligonucleotide intermediates () resolved on a non-denaturing polyacrylamide gel; (d) Detection of covalent protein-oligonucleotide intermediates assessed by SDS-PAGE. The arrowhead shows full length Tdp1-DNA and the lower band represents N-terminal proteolytic modified Tdp1 (NΔTdp1)-DNA intermediates. Numbers on top of the gel represent protein amounts in fmol. S: 14-mer oligonucleotide with 3'phosphotyrosyl modification (substrate), and P: 3'phosphoryl control oligonucleotide (product) containing same sequence as the 3'-pY substrate. A representative picture, of at least three independent experiments, is shown.

Fig. 8.

Kinetic analyses of wild type yeast Tdp1 and the SCAN1 (H432R) mutant. (a-c) Michaelis-Menten plots in which the reactions contained 65 fmol 5'-³²P labeled 3'-pY substrate combined with unlabeled substrate to the indicated final concentrations. Reaction velocities are shown as specific activity versus substrate concentration. (a) wild type Tdp1 (b) H432R and (c) H432R in which the Tdp1-DNA intermediate is regarded as a pseudo product from the experiment shown in B. (d) Single turnover experiment. The Tdp1-DNA covalent intermediate (I), the substrate (S) and the product (P) bands are labeled. (e) Time course of the H432R reaction. "o/n" denotes an aliquot that was incubated over night (> 16 hours). Bands are labeled as in (d).

Fig. 9.

Human Tdp1 mutants show a conserved phenotype. (a) Ten-fold serial dilutions of *top1Δtdp1Δ* cells co-transformed with YCpGAL1-hTOP1•U (↑hTOP1) and the indicated YCpGAL1-hTdp1•L or its control vector (*tdp1Δ*) were spotted onto selective galactose plates supplemented with or without sub-lethal CPT concentration (0.025 μg/ml) and incubated at 30 °C for 4 days. Human Tdp1 H493R and H493N are analogs of yeast Tdp1 H432R and H432N, respectively. A representative picture of at least three independent experiments is shown. (b) Total cell extracts from *top1Δtdp1Δ* cells co-transformed with vector control (*top1Δ*) and the indicated YCpGAL1-hTdp1•L or its control vector used in (a) were resolved on 4-12% SDS-PAGE and stained with anti-human Tdp1 (arrow), non-specific cross-reactive band (star). Blot stained with anti-αTubulin loading control (circle). (c) Partially purified total cell extracts of yeast and human N-terminally flag-tagged Top1 protein or control vector resolved on 4-12% SDS-PAGE and stained with anti-FLAG (M2) antibody.

Table 1

Data collection and refinement statistics

Data Collection	H182A	H432R	H432N	H432N_Glu
Wavelength (Å)	1.0	1.0	1.0	1.0
Space group	P1	P1	P1	P1
Unit Cell (Å/°)	64.15 81.79 98.59/ 86.92 85.53 67.10	63.87 81.49 96.79/ 89.52 84.90 67.82	64.45 82.04 98.68/ 89.80 94.33 112.78	64.35 82.19 99.29/ 86.79 85.57 66.32
Resolution Range	50-2.5	50-2.1	50-2.3	50-2.0
(MÅo)saicity (°)	0.633	0.948	0.623	0.314
Unique Reflections	69246	98077	75956	119602
Redundancy	2.2 (2.1)	3.5	3.2 (3.2)	1.9 (1.7)
Completeness (%) ^a	96.6 (95.3)	93.4 (74.5)	92.8 (97.3)	95.9 (81.3)
I/σ ^b	10.1 (2.2)	20.4 (3.6)	13.8 (5.5)	24.7 (3.6)
R _{sym} (%) ^{a, c}	6.4 (43.5)	5.6 (28.7)	7.1 (24.1)	6.7 (35.7)
Refinement				
Protein Atoms	13681	14230	13909	14684
Solvent Atoms	0	472	125	745
Ions and Ligand	0	16	-	35
R _{crystal} / R _{free} (%) ^{a, d}	20.0/ 22.7	18.89 / 22.34	20.42 / 23.58	17.60 / 21.28
RMS Bond Length	(32.03. 0/ 2 312.7)	(23.09. 0/ 2235.1)	(22.01. 0/ 2235.0)	(18.08. 0/ 2 244.7)
RMS Bond Angles	1.799	1.907	1.907	0.1958
RMS chirality	0.123	0.130	0.134	0.144

^aValues in parentheses are from the highest resolution shell.^bI/σ is the mean reflection intensity divided by the estimated error.^cR_{sym} = (Σ|I_{hkl} - <I>|)/(ΣI_{hkl}), where the average intensity <I> is taken over all symmetry equivalent measurements and I_{hkl} is the measured intensity for any given reflection.^dR_{crystal} = ||F_O - |F_C||/|F_O|, where F_O and F_C are the observed and calculated structure factor amplitudes, respectively. R_{free} is equivalent to R_{crystal}, but calculated for 5% of the reflections chosen at random and omitted from the refinement process.

Table 2

Calculated pKa values for selected residues

Structure		His182 ^a	His394	His432 ^a
Apo	wt-PO ₃ ^b	N.A.	7.3	5.8 (δ)
	wt	< 0.0	6.5	< 0.0 (δ)
	H432N	< 0.0	6.5	N.A.
	H432R	< 0.0	4.7	N.A.
DNA-pMP bound	wt	2.9	7.2	12.8 (ε)
	H432N	15.2	6	N.A.
	H432R	3.5	6.2	N.A.

^aDeprotonated His182 always prefers δ tautomer. Preferred tautomer of 432 as indicated.^bPhosphorylated His182.

Table 3

Kinetic constants for Tdp1 and H432R enzyme

	k_{cat} (min⁻¹)	K_m (mM)	k_{cat}/K_m (M⁻¹ s⁻¹)
Tdp1 Wild Type Tdp1 H432R	860 ± 25	114 ± 13.1	1.2 × 10 ⁸
Product	7.1 ± 0.60	2140 ± 300	5.5 × 10 ⁴
Intermediates	0.42 ± 0.015	148 ± 20.7	4.7 × 10 ⁴