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Human Tdp1 Cleaves a Broad Spectrum of Substrates Including Phosphoamide Linkages*

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Abstract

Human tyrosyl-DNA phosphodiesterase (Tdp1) hydrolyzes the phosphodiester bond between a DNA 3′ end and a tyrosyl moiety. In eukaryotic cells this type of linkage is found in stalled topoisomerase I-DNA covalent complexes and Tdp1 has been implicated in the repair of such complexes *in vivo*. We confirm here that the Tdp1 catalytic cycle involves a covalent reaction intermediate in which a histidine residue is connected to a DNA 3′ phosphate through a phosphoamide linkage. Surprisingly, this linkage can be hydrolyzed by Tdp1 and unlike a topoisomerase I-DNA complex, which requires modification to be an efficient substrate for Tdp1, the native form of Tdp1 can be removed from the DNA. The SCAN1 neurodegenerative disease is caused by the H493R mutant form of Tdp1 which shows reduced enzymatic activity and accumulates the Tdp1-DNA covalent intermediate. The ability of wt Tdp1 to remove the stalled mutant protein from the DNA likely explains the recessive nature of SCAN1. In addition to its activity on phosphotyrosine and phosphohistidine substrates, Tdp1 also possesses a limited DNA and RNA 3′ exonuclease activity in which a single nucleoside is removed from the 3′ hydroxyl end of the substrate. Furthermore, Tdp1 also removes a 3′ abasic site and an artificial 3′ biotin adduct from the DNA. In combination with earlier data showing that Tdp1 can use 3′ phosphoglycolate as a substrate, these data suggest that Tdp1 may function to remove a variety of 3′ adducts from DNA during DNA repair.

Tyrosyl-DNA phosphodiesterase (Tdp1)[1] was discovered as an enzymatic activity from *Saccharomyces cerevisiae* that specifically hydrolyzes the phosphodiester linkage between the O-4 atom of a tyrosine and a DNA 3′ phosphate (1). This type of linkage is typical of the covalent reaction intermediate produced when a type IB topoisomerase cleaves one strand of DNA. Type IB topoisomerases are ubiquitous enzymes that perform essential functions in key cellular processes such as replication, recombination and transcription (2-6). Following cleavage and DNA relaxation, the topoisomerase normally religates the DNA strand and dissociates from the DNA (4). After cleavage near certain DNA lesions or modified nucleotides, or in the presence of the anti-cancer drug camptothecin, religation is blocked and the topoisomerase becomes covalently trapped on the 3′ end of the cleaved DNA strand (7-10). At the time of the discovery of Tdp1, Yang et al. (1) suggested that the enzyme might be involved in the removal of such covalently stalled topoisomerase I molecules from the DNA. At present, extensive genetic, biochemical, and cell biological data support the view that Tdp1 is involved in the repair of topoisomerase I-DNA covalent lesions in eukaryotic cells (11-16).

Tdp1 is a member of the phospholipase D (PLD) superfamily which is characterized by two “HKD” motifs that provide the conserved active site residues (17). The diverse members of

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[1]The abbreviations used are: Tdp1, tyrosyl-DNA phosphodiesterase; topo70, NH_2-terminal truncation of human topoisomerase I missing first 174 amino acids; PNK, T

this family fulfill a variety of functions on vastly different substrates, such as phospholipids, DNA, or in the case of Tdp1, DNA-protein complexes (17-21). Based on structural and biochemical data, the conserved PLD superfamily reaction mechanism has been proposed to consist of two consecutive SN2 substitutions. In the first step of the Tdp1 reaction, a histidine residue in the N-terminal HKD motif (His263) is proposed to carry out a nucleophilic attack on the phosphorus atom in the phosphodiester bond between the tyrosine residue and the DNA 3′ oxygen (see Fig. 2B) to become covalently bound to the 3′ end of the DNA via a phosphoamide bond to the Nε2 atom of the nucleophilic His263 ((15,17,22-24) and see Fig 2C). Hydrolysis of this reaction intermediate is believed to be carried out by a water molecule that is activated by His493 from the C-terminal HKD motif acting as a general base. Tdp1 catalysis produces free tyrosine and a DNA with a 3′ phosphate end.

Recently, a mutation in the human *TDP1* gene, resulting in a change of His493 to arginine, has been shown to cause the heritable neurodegenerative disorder spinocerebellar ataxia with axonal neuropathy (SCAN1) (25). Affected individuals who carry the mutation in both *TDP1* alleles generally develop symptoms during their teenage years whereas heterozygous carriers remain symptom free. Interestingly, the SCAN1 condition does not seem to be associated with increased cancer susceptibility. Contrary to earlier predictions that the mutant enzyme is inactive (12,25,26), the overall enzymatic activity of H493R is reduced only about 25 fold (15). Importantly, this mutation causes an accumulation of the covalent Tdp1-DNA reaction intermediate which we have shown decays with a half-life of 13 min *in vitro* (15). Ironically, the covalent Tdp1 H493R-DNA complex replaces the covalent topoisomerase I-DNA complex that the mutant form of Tdp1 attempts to repair. Since SCAN1 cells contain substantial amounts of mutant Tdp1 (12,15), this finding suggests that the SCAN1 phenotype may not be caused by a mere reduction of enzymatic function alone, but also by the accumulation of Tdp1-DNA complexes. These persistent long-lived Tdp1-DNA and topoisomerase I-DNA complexes could significantly impact transcription in the terminally differentiated non-dividing neuronal cells that are primarily affected in SCAN1.

Based on our current understanding of the H493R mutant Tdp1, one would predict that the mutant Tdp1-DNA complexes must also occur in heterozygous carriers. The fact the SCAN1 mutation is completely recessive suggests that heterozygous, but not homozygous individuals possess an enzymatic activity that is able to remove Tdp1 from the DNA. The most likely source of this activity is wt Tdp1 itself and this possibility prompted us to test whether Tdp1 is able to act on the phosphoamide linkage that connects Tdp1 with the DNA in the covalent intermediate.

Previous work has established that Tdp1 can hydrolyze a variety of groups that are covalently linked to the 3′ end of DNA. In addition to a single tyrosine moiety, Tdp1 can also remove short peptides containing a tyrosine (17,27). Interestingly, an intact topoisomerase I-DNA complex has proven to be a very poor substrate for yeast Tdp1 *in vitro* (27). It has been suggested that the stalled topoisomerase is proteolytically degraded or otherwise modified before Tdp1 action *in vivo* (1,23,28,29). In addition to its cleavage activity on a 3′ phosphotyrosyl linkage, Tdp1 has been shown to cleave the phosphodiester bond linking glycolate to the 3′ end of DNA (30). Such a 3′ phosphoglycolate adduct is formed when cells are exposed to ionizing radiation or the drug bleomycin, or as a result of naturally occurring endogenous reactive oxygen species (31). In addition to these naturally occurring substrates, several synthetic Tdp1 substrates have been identified such as the phosphotyrosine analogs, 3′-(4-nitro-phenyl) phosphate, (4-methyl-phenyl)-phosphate, and 4-methylumbelliferone which proved helpful in dissecting the catalytic mechanism of Tdp1 (24,32).

In this study, we confirm chemically that the covalent Tdp1-DNA intermediate is indeed a phosphoamide linkage as had been proposed based on both structural and biochemical data

(17,23,33). Surprisingly, Tdp1 is not only able to cleave a phosphoamide bond efficiently, but is also able to remove an intact Tdp1 molecule from the DNA *in vitro*. Furthermore, we used several additional Tdp1 substrates to demonstrate novel Tdp1 activities *in vitro*. These results support the possibility that Tdp1 might have additional cellular functions besides its role in the repair of topoisomerase I-DNA lesions.

EXPERIMENTAL PROCEDURES

Mutagenesis, Expression, and Purification of Tdp1 Proteins—The expression plasmid for the N-terminally truncated H493R mutant form of human Tdp1(Δ(1-148) H493R) was generated by site-directed mutagenesis using plasmid pHN1894S Δ(1-148) which contains the *TDP1* gene lacking the first 444 base pairs (17). The generation of the expression plasmids for the other mutant alleles has been described previously (15,17). The N-terminally His-tagged recombinant Tdp1 proteins (wt, Δ(1-148), H493R, Δ(1-148) H493R, and H263A) were expressed in *E. coli* BL21 (DE3) and purified as described previously (17).

Preparation of Tdp1 Substrates—Substrate 20-TOPOpep, a 20-mer DNA oligonucleotide (5′-GTAGAGGATCTAAAAGACTT-3′) with a small trypsin-resistant human topoisomerase I-derived peptide covalently bound to the 3′ end via the active site tyrosine, was prepared as described previously (17). Briefly, a duplex 30-mer topoisomerase I suicide substrate consisting of the cleavage strand HEI-9 (5′-GTAGAGGATCTAAAAGACTT*TGAAAA ATTT, *indicates a 5′-bridging phosphorothiolate linkage at the topoisomerase cleavage site (34)) and the complementary strand HEI-10 (5′-AAATTTTTCAAAGTCTTTTAGATCCTCTAC) was incubated with topo70 (a 70 kDa form of human topoisomerase I that is missing the nonessential N-terminal 174 amino acids) for 15 min at 37°C to allow suicide cleavage and covalent binding of topo70 to the remaining 20-mer oligonucleotide (20-Topo). Subsequent trypsin treatment and gel purification resulted in a 20-mer single-stranded oligonucleotide with a predicted 7 amino acid topoisomerase I-derived peptide.

Substrate 20-TDPpep was generated by incubating 0.05 pmol of 20-TOPOpep with 0.2 μg Tdp1 H493R in Assay Buffer (100 mM KCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT) for 2 min at 37°C and stopping the reactions with 0.5% SDS. Under these conditions more than 50% of the labeled DNA is trapped in the covalent complex with Tdp1 (see Fig. 2A, lane 2). The reactions were diluted 10-fold with TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) and trypsin was added to a final concentration of 1.5 μg/μl. Samples were incubated for 2 h at 37°C. Trypsin was inactivated by the addition of 1 μg/μl PMSF. The resulting 20-TDPpep is predicted to consist of an 11 amino acid Tdp1-derived peptide linked to the 3′ end of the 20-mer DNA.

The sequences of the 20-mer single-stranded DNA oligonucleotides 20-Y and HEI-23 are identical to the oligonucleotide portion of 20-TOPOpep but end with a 3′ tyrosyl moiety or a hydroxyl, respectively, in place of the peptide. The 3′ biotinylated oligo HEI-22 is complementary to 20-Y. The 19-mer DNA oligonucleotide HEI-24 (5′-GTAGAGGATCTAAAAGACT-3′) is 3′ phosphorylated. The 29-mer RNA oligonucleotide Md9 has the following sequence: 5′-ACCCCACCUGUAGGUUUGGCAAGCUAGCU-3′ and was annealed to the 48-mer DNA oligonucleotide D49Δ8 (5′-CTAGCTTGCCAAACCTACAGGTGGGGTCCCCCCTTTTCTGGAGACTAA-3′) after 5′ end-labeling (35). The 20-mer DNA oligonucleotide HEI-22 (5′-AAGTCTTTTAGATCCTCTAC-3′) has a biotin moiety connected by a 15-atom spacer on the 3′ end (BioTEG). The 3′ phosphorylated 20-mer oligonucleotide HEI-27 is identical in

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sequence to HEI-22. The 20-mer DNA oligonucleotide HEI-28 is identical in sequence to HEI-23 but contains an additional abasic site mimic (tetrahydrofuran) at the 3′ end.

All DNA oligonucleotides except HEI-9 (Macromolecular Resources) and 20-Y (Midland) were synthesized by OPERON. All substrates were ^{32}P 5′-end-labeled with T4 polynucleotide kinase (PNK) (New England Biolabs). In addition, 3′ phosphorylated oligonucleotides were ^{32}P 5′-end-labeled labeled using a 3′ phosphatase minus PNK (MBI Fermentas).

Phosphoamino Acid Lability Analyses—To create a suitable substrate to study the nature of the phosphoamino acid linkage between Tdp1 and the 3′ end of the DNA, oligonucleotides CL12N (5′-GAAAAAAGAGTT-3′) and CP25N (5′-TAAAAATTTTTCTAACTCTTTTTTC-3′) were annealed and Klenow (3′-5′ exo$^-$) (NEB) was used to generate a 14-mer topoisomerase I suicide substrate by fill-in in the presence of [α-^{32}P]dATP and ddGTP (see Fig. 1A). 50 pmol of this substrate were incubated with 19 μg topo70 in 200 μl of reaction buffer (150 mM KCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT) at room temperature for 45 min to generate the topoisomerase I-DNA cleavage complex consisting of a 12-mer oligonucleotide with the topoisomerase covalently bound to the 3′ end (12-Topo). Part of this reaction was stopped with SDS (final concentration 1%) and used for a parallel analysis of the topoisomerase I-DNA linkage. The other part was digested with trypsin, the trypsin was inactivated with PMSF, and the product was gel purified to generate 12-TOPOpep. In this complex the phosphate that is in involved in the phosphotyrosyl linkage between the DNA and the topoisomerase I peptide is radioactively labeled. To accumulate the covalent Tdp1-DNA reaction intermediate (12-Tdp1), 0.05 pmol of 12-TOPOpep were incubated with 0.2 μg Tdp1 H493R in 25 μl of Assay Buffer at 37°C for 3 min. The reaction was stopped by the addition of SDS to a final concentration of 1%. Again, the phosphate involved in the phosphoamino acid DNA linkage between Tdp1 and the DNA is radioactively labeled.

For the phosphoamino acid lability experiment (36,37) the labeled 12-Tdp1 was incubated in 1% SDS at 65°C for 10 min to denature the protein. At that time a 5 μl control sample was taken and boiled in SDS sample buffer for gel analysis (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromphenol blue). To a series of 10 μl aliquots of the treated 12-Tdp1 were then added 2.5 μl each 1 M Tris-HCl, pH 7.5, 1 M HCl, 1 M NaOH or 1M hydroxylamine in 200 mM Tris-HCl, pH 7.5, and the reactions were incubated at 37°C for 10 min. Subsequently, 4 μl of 5$_x$ SDS sample buffer were added to each reaction. To neutralize the acid and base treated samples 2.5 μl of 1 M NaOH or 1 M HCl were added respectively. To the reactions that were incubated in buffer alone or with hydroxylamine, 2.5 μl of 1 M Tris-HCl, pH 7.5, or 200 mM Tris-HCl, pH 7.5, were added respectively. Samples were boiled for 3 min and analyzed by 10% SDS-PAGE. Gels were dried and image retrieval was carried out using a PhosphorImager and ImageQuant software (Amersham Biosciences). As a control 12-Topo in which the DNA is linked to the protein via a phosphotyrosyl bond was treated identically.

Tdp1 Activity on Phosphoamide Bond—A sample containing >50% 20-TDPpep (see Preparation of Tdp1 substrates and Fig. 2A, lane 2) was incubated with 50 ng wt or H263A Tdp1 in Assay Buffer (final volume 10 μl). After a 30 min incubation at 37°C, reactions were stopped with an equal volume of formamide loading dye (96% formamide, 20 mM EDTA, 0.03% xylene cyanol, 0.03% bromphenol blue) and analyzed on a 15% sequencing gel. Image retrieval and quantitation were carried out as described above.

Tdp1 Activity on intact Topo70 or Tdp1 Covalently Bound to DNA—To generate the topo70-DNA covalent complex, 2.5 pmol of suicide substrate HEI-9/HEI-10 was incubated with 1 μg topo70 in 20 μl of Assay Buffer for 30 min at 37°C. One third of the reaction was

subsequently incubated at 65°C for 10 min. To the second third of the reaction, SDS was added to a final concentration of 0.07%. The last third of the reaction mix was left untreated. To 0.2 pmol of each of these three substrates, 400 ng wt Tdp1 or the H263A mutant Tdp1 was added in a total volume of 60 μl in Assay Buffer and incubated at 37°C. At the indicated time points (Fig. 3A), 15 μl samples were taken and the reactions were stopped with SDS (0.5% final concentration). Samples were precipitated in 10 mM $MgCl_2$ with 5 volumes of ethanol in the cold. The dry pellets were resuspended in 8 μl of 2 μg/μl of trypsin and incubated at 37°C for 2 h. Samples were mixed with 12 μl formamide loading dye and the reactions were analyzed on a 15% sequencing gel. For purposes of quantitation, the amount of product at each time point was determined as a percentage of the total radioactivity in the lane of the gel.

To generate the Tdp1 covalent reaction intermediate, 0.05 pmol of 20-TOPOpep were incubated with 0.2 μg Tdp1-H493R in 25 μl Assay Buffer at 37°C for 2 min and the reactions stopped by the addition of SDS to 0.5%. Part of the reaction mixture was digested with trypsin as described above to determine the amount of H493R Tdp1 covalent intermediate (20-Tdp1) as reflected by 20-TDPpep in a sequencing gel analysis. 0.5 μl of the untreated reaction mixture was incubated with 50 ng wt or H263 Tdp1 in Assay Buffer (total volume 10 μl) and the samples were incubated at 37°C for 30 min before stopping with an equal volume of formamide loading dye. Sequencing gel analysis, image retrieval and quantitation were carried out as described above.

Fate of H493R-DNA Intermediate in the Presence of Various Forms of Tdp1— A 50-fold molar excess of either full length H493R or the N-terminally truncated Δ(1-148) H493R (0.1 μM) was incubated with 20-TOPOpep (2 nM) for 2 min at 37°C in Assay Buffer (final volume 25 μl) to allow for the accumulation of the covalent enzyme-DNA intermediate. An equal volume of Assay Buffer containing the indicated form of Tdp1 was added (final concentration 0.25 μM). Parallel control incubations were carried out without added enzyme. At the indicated time points, samples were stopped by the addition of SDS to 0.5%. Subsequent SDS-PAGE analysis, or trypsin digestion followed by sequencing gel analysis was carried out as described above.

Determination of the Half-life of the DNAH493R Tdp1 Covalent Complex in the Absence of Free Tdp1—5′ end-labeled 20-Y was annealed to the 3′ biotinylated DNA oligonucleotide HEI-22 and 0.05 pmol of the fully duplexed 20-mer was incubated with streptavidin conjugated paramagnetic beads (Streptavidin MagneSphere, Promega) equilibrated in Assay Buffer to allow complete binding of the Tdp1 substrate to the beads. A 50-fold molar excess of H493R Tdp1 (0.1 μM) was added and the 25 μl reaction was incubated for 1 min at 37°C. At this point the beads were washed twice with 500 μl Assay Buffer to remove free Tdp1. Elimination of free enzyme after the washes was verified by testing for Tdp1 activity in the supernatant (data not shown). Subsequently the beads were resuspended in approximately 50 μl of Assay Buffer and 5 μl aliquots removed at the indicated time points and the reactions stopped with 0.5% SDS. After a 10-fold dilution with TE, the samples were digested with trypsin, and analyzed on a 15% denaturing sequencing gel as described above.

Tdp1 Activity Assays with DNA, RNA, and 3′ Biotinylated DNA—A 20-mer DNA oligonucleotide containing a 3′ hydroxyl (0.05 pmol HEI-23) was 5′ end-labeled and incubated with 25-100 ng of wt or H263A Tdp1 in 10 μl Assay Buffer at 37°C for 15-30 min and reactions were stopped with an equal volume of formamide loading dye. Where indicated, the samples were treated with PNK before the addition of formamide loading dye to remove potential 3′ phosphate groups. For the PNK treatment the reaction volumes were doubled to 20 μl by the addition of PNK reaction buffer (final concentrations: 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) and 30 U PNK and incubated for 30 additional min. As controls, the 5′ end-labeled and 3′ phosphorylated 19-mer oligonucleotide HEI-24 was incubated with 30 U

T4 PNK or 3′ phosphatase minus PNK in 20 μl PNK buffer at 37°C for 30 min. All reactions were stopped by the addition of an equal volume of formamide loading dye and samples were analyzed on a 15% sequencing gel.

The 5′-end-labeled 29-mer RNA oligonucleotide Md9 was annealed to a twofold excess of the 48-mer DNA oligonucleotide D49Δ8. 0.05 pmol of this substrate was incubated with 600 ng of either wt or H263A Tdp1 in 10 μl Assay Buffer at 37°C for 15 min and reactions were quenched with formamide loading dye. As control markers for 3′ hydroxyl and 3′ phosphate RNAs, P1 nuclease (Roche) and NaOH ladders of Md9, respectively, were generated. 0.05 pmol of the 3′ biotinylated 20-mer DNA oligonucleotide HEI-22 and the 3′ abasic site oligonucleotide HEI-28 were incubated for 15 min with either 25 ng of wt or H263A Tdp1 and treated as described above for HEI-23.

RESULTS

Tdp1 is Linked to the DNA in the Covalent Reaction Intermediate Via a Phosphoamide Bond—Biochemical and structural data point to His263 as the nucleophile in the first SN2 reaction in the catalytic cycle of Tdp1 (17,23,33). In particular the crystal structures show His263 bound covalently to the phosphate analog vanadate in a mimic of the transition state of the reaction. To obtain independent evidence that the linkage between the DNA and Tdp1 is indeed a phosphoamide linkage, the chemical stability of the Tdp1-DNA linkage was tested.

It was essential that the very phosphate involved in the enzyme-DNA linkage was labeled in the substrate used for the chemical analysis. Therefore a 12-mer DNA oligonucleotide was annealed to a 25-mer and filled in with [α -^{32}P]dATP and ddGTP to generate a suicide substrate for human topoisomerase I (Fig. 1A). Upon cleavage the topoisomerase remains covalently bound to the 3′ end at the cleavage site because the 3′ dinucleotide (AG) dissociates after cleavage and is unavailable for religation. At this point the labeled phosphorus is in the phosphodiester bond between the DNA and Tyr723 of the topoisomerase. Subsequent trypsin digestion removes most of the protein and leaves a 7 amino acid peptide on the DNA. This labeled 12-mer DNA with the topoisomerase peptide (12-TOPOpep) was used as a substrate for the Tdp1 H493 mutant. This mutant enzyme accumulates the covalent intermediate to high levels thus enabling experiments to study the nature of the covalent DNA-Tdp1 intermediate. The Tdp1-DNA intermediate (12-Tdp1) could be visualized on SDS-PAGE since the protein was radioactively labeled via the ^{32}P in the DNA substrate (Fig, 1B, lane 1). In contrast to O-phosphate linkages which are relatively resistant to treatment with acid, base and hydroxylamine, N-phosphate linkages are resistant to base, but sensitive to acid treatment and hydroxylamine (36-38). The Tdp1-DNA intermediate proved stable in NaOH (Fig. 1B, lane 4), but sensitive to acid (lane 3) and hydroxylamine (lane 5) treatment confirming that the linkage does indeed involve a phosphoamide bond. As a control for the method, the covalent topoisomerase I-DNA complex (12-Topo) was treated identically and the P-O phosphotyrosyl bond between the topoisomerase and the DNA proved resistant to all conditions tested as expected (Fig. 1C).

Tdp1 Can Cleave the Phosphoamide Bond Between a Tdp1-derived Peptide and the 3′ End of DNA—We were interested in testing the possibility that wt Tdp1 is capable of removing the covalently trapped H493R mutant Tdp1 protein from the DNA in heterozygous carriers of the SCAN1 H493R Tdp1 mutation. To test this hypothesis, we first asked if wt Tdp1 could hydrolyze a phosphoamide bond. To generate a suitable substrate, we started with a 20-mer DNA oligonucleotide containing a human topoisomerase I-derived peptide on the 3′ end (20-TOPOpep, Fig. 2A, lane 1 and Fig. 2B) and incubated the DNA with Tdp1 H493R for a short time to allow the Tdp1-DNA covalent intermediate to accumulate. The reaction was stopped with SDS and the products were treated with trypsin to degrade most of the Tdp1 in the complex

and leave a predicted 11 amino acid peptide attached to the 3′ end of the DNA by a phosphoamide bond. Sequencing gel analysis showed that under these conditions approximately 70% of the label was in the intermediate (20-TDPpep, Fig. 2C) with the remainder having been converted into product, a 20-mer with a 3′ phosphate (20-P) (Fig. 2A, lane 2). This mixture was used as a substrate in reactions with wt Tdp1 and, as a control, with the inactive mutant H263A (17). Wt Tdp1 completely removed the tryptic Tdp1 peptide from the DNA (lane 3) whereas the inactive mutant enzyme left the substrate unchanged (lane 4). These data demonstrate that wt Tdp1 can cleave the phosphoamide bond between the DNA 3′ end and the histidine in the Tdp1 peptide.

Partially Denatured Topoisomerase I-DNA and Tdp1-DNA Covalent Complexes Are Tdp1 Substrates—In the initial study describing the Tdp1 activity, it was reported that the intact protein-DNA complex (in this case formed using the λ integrase) was a relatively poor substrate for *S. cerevisiae* Tdp1 as compared with a tyrosine-DNA complex (1). Because a brief heat treatment converted the complex into a much better substrate, it was suggested the protein bound to the DNA may need to undergo a conformational change or partial degradation in order to become accessible as a substrate for Tdp1. Furthermore, the phosphodiester bond between the human topoisomerase I nucleophilic Tyr723 and the DNA is buried deep within the topoisomerase I clamp around the DNA and appears relatively inaccessible (39,40).

To test the ability of human Tdp1 to hydrolyze the tyrosyl-phosphodiester bond between an intact topoisomerase and the DNA we used a different kind of suicide substrate, a duplex 30-mer DNA substrate with a bridging phosphorothiolate at the preferred topoisomerase I cleavage site. After topoisomerase cleavage at that position, the sulfhydryl group at the 5′ end of the cleaved strand is unable to act as a nucleophile for the second step of the topoisomerase reaction and therefore religation is prevented (34). An excess of topo70, a fully functional N-terminal truncation of human topoisomerase I, was incubated with this suicide substrate so that virtually all of the DNA became covalently bound to the enzyme. This covalent complex was used as a substrate for Tdp1 before and after various treatments. In order to visualize the covalent topoisomerase I-DNA complexes which are unable to enter a 15% sequencing gel, the Tdp1 reaction products were treated with trypsin prior to sequencing gel analysis to convert all of the unreacted 20-Topo to 20-TOPOpep (Fig. 3, lanes 3-13). When Tdp1 was added to the untreated intact topoisomerase I-DNA complexes, only a small percentage of the substrate was cleaved to produce 20-P during the time course experiment shown in Fig. 3A, lanes 3-6. Heating of the covalent complexes for 10 min at 65°C prior to Tdp1 addition led to an increase in the percentage of the DNA converted to product, which interestingly did not increase over the course of the experiment (Fig. 3A, lanes 7-10) possibly because some the protein remained folded after the heat treatment. Treatment with SDS converted the topoisomerase-DNA complex into a good substrate for wt Tdp1 (Fig. 3A, lanes 11 and 12) after the sample had been diluted to reduce the SDS concentration to a level tolerated by Tdp1 (0.0025% SDS). As expected, the inactive Tdp1 H263A mutant did not cleave the substrate even in the presence of SDS (Fig. 3A, lane 13). These data emphasize that the topoisomerase I must be at least partly denatured in order to be a suitable substrate for Tdp1.

Based on our previous observation (see above) that Tdp1 can hydrolyze the phosphoamide bond between a Tdp1 tryptic peptide and the DNA, we wondered whether full length SDS-denatured Tdp1-DNA complex would also be a substrate for Tdp1. Because no suicide substrate analogous to the ones described for topoisomerase I have been identified for Tdp1, we employed the H493R mutant protein which accumulates the Tdp1-DNA covalent reaction intermediate as described above. The H493R mutant was incubated with 20-TOPOpep, a single stranded 20-mer DNA with a topoisomerase tryptic peptide at the 3′ end and the reaction was stopped with SDS (Fig. 3B, lane 1). At this point approximately 50% of the labeled substrate DNA was in the covalent complex with Tdp1 H493R as revealed by the trypsin-digested sample

loaded in Fig. 3B, lane 4 (20-TDPpep). A small amount of the substrate 20-TOPOpep was still visible and the remainder of the substrate was already converted to product (20-P). Except for the trypsin treatment, the sample shown in lane 4 is identical to the one loaded in lane 1 in which the Tdp1-DNA complex cannot be seen because it remained trapped in the well of the gel. Addition of wt Tdp1 to this substrate mix resulted in the conversion of all labeled species to the product 20-P (Fig. 3B, lane 2). Since the increase in the intensity of the product band cannot be accounted for by the conversion of the weak 20-TOPOpep substrate band alone, we conclude that the full length Tdp1-DNA covalent complex after SDS treatment must also have been cleaved. Furthermore, the sum of label in the three bands in lane 4 is identical to the amount of label in the product band in lane 2. In contrast, the inactive H263A mutant enzyme had no effect on the substrate (lane 3). Based on these results, we conclude that Tdp1 is able to remove both intact topo70 and Tdp1 efficiently from the DNA 3′ end after denaturation by SDS.

Tdp1 Can Remove Native Tdp1 from the DNA—The finding that Tdp1 can catalyze the hydrolysis of the phosphoamide bond between the 3′ end of the DNA and a Tdp1 peptide containing His263 or even a full-length SDS-denatured Tdp1 molecule prompted us to ask whether Tdp1 is capable of removing the entire native Tdp1 protein as well. Excess H493R was incubated with substrate 20-TOPOpep for 2 min to ensure that none of the substrate remained and that all of the radioactivity was either in the product 20-P or the covalent intermediate 20-Tdp. Treatment of a portion of the reaction with trypsin at this time point and analysis on a sequencing gel confirmed that the substrate was exhausted and that more than half of the label was associated with the covalent intermediate (20-TDPpep) (Fig. 4A, lane 3 and Fig. 4B, lane 2). At the indicated time points after the initial 2 min incubation, samples were removed from the reaction mixture, stopped with SDS, and treated with trypsin to visualize the covalent DNA-Tdp1 complexes (Fig. 4B, lanes 3-7). As we had observed previously (15), over the course of the experiment the covalent intermediate slowly disappeared and there was a corresponding increase in the amount of product 20-P. Addition of more H493R did not change the rate of product formation (compare lanes 3-7 with 13-17, and see Fig. 4C). When a 5-fold excess of wt Tdp1 was added after the initial 2 min incubation with H493R, the rate of product formation increased at least 5-fold (Fig. 4B, lanes 8-12, and Fig. 4C) indicating that wt Tdp1 either stimulated hydrolysis of the covalent reaction intermediate by the bound H493R enzyme or, alternatively, that wt Tdp1 actively removed the covalently bound native form of H493R from the DNA. The fact that the addition of the inactive mutant enzyme H263A did not have a stimulatory effect (Fig. 4B, lanes 23-27, and Fig. 4C) suggested that wt Tdp1 was able to remove the intact native H493R protein from the DNA.

To test if the N-terminus of Tdp1 was required for removal of the stalled H493R mutant enzyme from the DNA, we tested an N-terminal truncation mutant which is missing the first 148 amino acids, but retains full enzymatic activity. Results with the truncated protein were indistinguishable from those obtained with full-length Tdp1 (compare Fig. 4B, lanes 8-12 with lanes 18-22, and see Fig. 4C). Thus, it is unlikely that the N-terminus plays a critical role in the removal of the full-length Tdp1 enzyme.

The H493R Mutant Transfers the DNA Substrate from One Enzyme Molecule to Another—In order to formally show that Tdp1 can remove intact stalled H493R, we performed experiments essentially the same as those described above (Fig. 4), but the fate of the trapped 20-Tdp1 intermediate was monitored over time by both SDS-PAGE (Figure 5A) and sequencing gel analysis (not shown). The possibility that the covalent Tdp1 H493R-DNA intermediate that accumulates in the H493R reaction is itself a substrate for Tdp1 cleavage raises the interesting possibility that the labeled DNA in the intermediate is successively transferred from one mutant enzyme to another. To test whether this type of DNA “hand-off” occurs, we incubated H493R with 20-TOPOpep for 2 min to accumulate the covalent intermediate and then added an N-

terminally truncated Tdp1 H493R variant missing the first 148 amino acids that are dispensable for activity (Δ(1-148) H493R). Covalent reaction intermediates formed with full-length H493R can be distinguished from those formed with the truncated H493R enzyme by their different mobilities in SDS-PAGE (Fig. 5A). Transfer of the labeled DNA from the covalent intermediate formed with the full length enzyme (20-Tdp FL) to the faster-migrating truncated form of the enzyme (20-Tdp Δ) can be seen after addition of the deletion variant (Figure 5A, lanes 28-36). Conversely, allowing the substrate to react with Δ(1-148) H493R first, followed by the addition of full length H493R produced the reciprocal result (Figure 5A, lanes 37-45). These results using the various forms of the H493R mutant enzyme firmly establish that the DNA moiety of the covalent intermediate can be transferred from one enzyme molecule to another.

Since H493R can access the phosphoamide bond between the native H493R enzyme and the DNA, then wt Tdp1 should also be able to remove the mutant protein from the DNA. In fact, addition of wt protein reduced the apparent half-life of the covalent intermediate to less than 2 min (Fig. 5A, lanes 1-9, and Fig. 5B, open circles), as compared to 13 min for H493R alone (data not shown) or after the addition of excess mutant H493R enzyme (Fig. 5A, lanes 19-27 and Fig. 5B, open squares). Active Tdp1 was required for this reaction since addition of the inactive H263A mutant protein to the H493R covalent intermediate produced results that were indistinguishable from those obtained by the addition of excess H493R to the reaction (Figure 5A, compare lanes 19-27 with lanes 10-18). These data confirm the remarkable finding that Tdp1 can cleave the DNA from an intact native form of the Tdp1 H493R protein.

While the trapped intermediate predominantly appeared as one distinct band on sequencing gels (see Fig. 4B), two bands were consistently observed for both the full length and Δ(1-148) H493R truncation mutant by SDS-PAGE (Figure 5A). We do not know the basis for the doublet, but two species with different mobilities in SDS-PAGE were not observed in a similar analysis of the purified enzyme used for the experiments (data not shown). Since the relative amounts of the two bands varied slightly from experiment to experiment, the sequencing gel data where only a single band was present were used for the quantitation shown in Fig. 5B.

The Half-life of the DNA-Tdp1 H493R Covalent Complex is the Same in the Presence and Absence of Excess Enzyme—We proposed earlier (15) that the accumulation of the covalent H493R-DNA intermediate with an apparent half-life of 13 min might have an impact on the phenotype of SCAN1 patients. In view of the fact that the biochemical experiments to measure the half-life were done with a 50-fold excess of enzyme over substrate and the surprisingly rapid hand-off observed in Fig. 5A (lanes 28-45), we wondered whether the actual half-life of the DNA-H493R covalent complex might be different in the absence of free Tdp1 H493R enzyme. To answer this question, the 5′ end-labeled DNA oligonucleotide 20-Y containing a 3′ tyrosyl moiety, was annealed to a complementary 3′ biotinylated oligonucleotide (HEI-22) and the 20-mer duplex DNA was bound to streptavidin coated paramagnetic beads. The immobilized substrate was incubated with a 50-fold molar excess of H493R Tdp1 for 1 min at which time all of the labeled substrate was either converted to the product 20-P or trapped in the covalent reaction intermediate. Free excess Tdp1 enzyme was quickly removed by extensive washing of the beads. In a time course experiment similar to the ones described above, samples were removed from the mixture and the reactions stopped with SDS. After trypsin digestion, the fate of the covalent intermediate was monitored over time using sequencing gel analysis. The rate of decay of the covalent intermediate shown in Fig. 5B (filled triangles) yielded a half-life for DNA-Tdp1 H493R complex in the absence of an excess of free enzyme of ~16 min which is very similar to the 13 min half-life observed in the presence of excess enzyme.

Tdp1 Displays Both DNA and RNA 3′ Exonuclease Activity—To test if Tdp1 can hydrolyze a phosphodiester bond in DNA, we incubated a 5′ end-labeled 20-mer DNA oligonucleotide containing a 3′ hydroxyl group with Tdp1. Wt Tdp1 but not the catalytically inactive H263A mutant Tdp1 removed one nucleoside from the 3′ end of the labeled DNA (Fig. 6A, lanes 4 and 5). The product of the reaction was a 19-mer with a 3′ phosphate as determined by comparison with a control 19-mer with a 3′ phosphate that had been 5′ end-labeled either with PNK (19-OH) or with 3′ phosphatase minus PNK (mixture of 19-OH and 19-P) (Fig. 6A, compare lanes 2 and 3 with 4). By comparison, cleavage of the DNA substrate requires approximately 50-times more enzyme than comparable cleavage of 20-TOPOpep (data not shown). Even upon prolonged incubation with wt enzyme, no further degradation of the 19-P oligonucleotide was observed which confirms that human Tdp1 cannot act on a DNA end containing a 3′ phosphate and furthermore is devoid of 3′ phosphatase activity. To verify independently that the product of the reaction indeed possessed a 3′ phosphate, PNK was added after incubation of the 20-mer with Tdp1. The 3′ phosphatase activity of PNK removed the 3′ phosphate from 19-P to produce a 19-mer with a 3′ hydroxyl (Fig. 6A, lane 7). In an otherwise identical control reaction with H263A neither the inactive Tdp1 nor PNK changed the mobility of the 20-OH substrate band. Attempts to target Tdp1 to an internal DNA phosphodiester bond using various “flap” structures, containing branch points between double- and single-stranded DNA, were unsuccessful (data not shown). In all cases tested, a mononucleoside was removed from the 3′ hydroxyl end of the flap.

Having established that Tdp1 has 3′ exonuclease activity on DNA, we next asked if the observed activity on DNA might extend to RNA. Therefore, a 29-mer RNA oligonucleotide was annealed to a longer complementary DNA oligonucleotide to prevent the formation of RNA secondary structures. The two 3′ most nucleotides of the RNA in this substrate extended beyond the DNA 5′ end to create a two nucleotide 3′ overhang. Incubation of this substrate with wt Tdp1 resulted in the removal of one ribonucleoside from the 3′ end of the RNA and generated a 28-mer RNA oligonucleotide with a 3′ phosphate as the product (Fig. 6B, lane 4). The control reaction with H263A confirmed that active Tdp1 was required for this reaction.

Tdp1 Acts on 3′ Abasic Sites and a 3′ Biotin Tag—To identify new 3′ DNA adducts that might represent substrates for Tdp1 we tested two additional oligonucleotide structures: a tetrahydrofuran moiety as stable abasic site mimic (Fig. 7A) and a biotin moiety connected to the DNA 3′ end via a 15 atom spacer (Fig. 7C). Both adducts were efficiently removed by wt Tdp1 to produce a 20-mer with a 3′ phosphate (20-P) while the inactive mutant H263A had no effect (Fig. 7B and D, lanes 4 and 5). Thus, cleavage occurred 3′ of the phosphate between the tetrahydrofuran moiety and the DNA or the 15 atom linker and the DNA, respectively,

DISCUSSION

Based on a combination of mutational, biochemical, and structural studies with other members of the PLD superfamily, it has been shown that a histidine in one of the HKD motifs in the enzyme is the nucleophile that attacks the scissile phosphate in the first step of the reaction to generate the covalent reaction intermediate (37,38). We have shown previously that a H263A mutation completely eliminates activity consistent with a similar role for this residue in Tdp1 catalysis (17). In addition, in co-crystal structures of human Tdp1 containing the transition state analog vanadate, His263 was found to be covalently bound to the vanadate (22,23), strongly suggesting that this amino acid is the nucleophile in the reaction. Because of its importance for the interpretation of the cleavage data presented here, we wanted to independently verify that the Tdp1 covalent intermediate that is generated using a natural substrate does indeed involve a phosphoamide linkage between the enzyme and a DNA 3′ phosphate. We took advantage of the fact that the H493R Tdp1 mutant enzyme accumulates the covalent intermediate to measure the chemical stability of the Tdp1-DNA linkage under a

variety of conditions. The lability profile matches the pattern for a phosphoamide linkage, involving either phosphohistidine or phospholysine, since the bond was sensitive to both acid and hydroxylamine, but resistant to treatment with alkali (36-38). These results combined with crystal structure data (22,23) and with our previous observations that both lysines within the active site can be mutated without eliminating catalytic activity (17) clearly establish that a phosphoamide bond links a histidine to the DNA in the covalent intermediate.

Using a tryptic peptide of Tdp1 H493R bound to the 3′ end of an oligonucleotide as a substrate for wt Tdp1, we found that the enzyme is capable of hydrolyzing the phosphoamide linkage between the peptide and the DNA. Thus, in addition to its known phosphodiesterase activity Tdp1 also possesses phosphoamidase activity. This reaction can be explained given our current understanding of the chemistry of catalysis by Tdp1. Based on crystal structures containing vanadate complexes (22,23,33), a well-defined constellation of amino acid side chains is involved in activating H263 for nucleophilic attack on the tyrosyl-3′ phosphate linkage and in stabilizing the pentavalent transition state. Nucleophilic attack by the same active site histidine on the phosphoamide bond in the Tdp1 covalent complex is predicted to follow the same chemistry with the only difference being the nature of the leaving group in the cleavage reaction. Unlike the case of the topoisomerase-derived tyrosine-DNA linkage where the leaving atom is the O-4 of tyrosine, in the case of the transamidation reaction involving the histidine-DNA linkage in the Tdp1 complex, the leaving atom is the Nε2 of the histidine. Notably, one other member of the PLD superfamily (cabbage PLD) has been reported to cleave a phosphoamide bond in an artificial substrate (41). However, this feature is not common to all PLD superfamily members since *Streptomyces* sp. PMF PLD is unable to cleave that substrate (41).

The ability of the Tdp1 to remove the DNA from a native Tdp1 H493R covalent intermediate is quite remarkable. For example, the *S. cerevisiae* and human Tdp1 activities are unable to efficiently catalyze the removal of the native form of human topoisomerase I when it is stalled in the covalent complex on DNA ((27) and Fig. 3A, lanes 3-6), likely because the substrate phosphodiester bond is buried deep within the topoisomerase I protein and therefore inaccessible to the Tdp1 active site (39,40). Denaturation of the protein moiety with heat or by the addition of SDS (Fig. 3A, lanes 7-12) renders the covalent complex a good substrate for Tdp1. Thus, it appears that the topoisomerase portion of the covalent complex must be cleaved by proteolysis or otherwise modified prior to cleavage by Tdp1. Indeed, topoisomerase I was found to be proteolytically degraded by the 26S proteasome in response to camptothecin treatment (28,29). Similar to the situation with topoisomerase I, an SDS denatured DNA-Tdp1 covalent complex is completely accessible to Tdp1 action. However, it was surprising to find that unlike the native topoisomerase I-DNA complex which is not a substrate for Tdp1, Tdp1 can remove a fully-folded Tdp1 enzyme from the DNA. It is not obvious how the native form of the Tdp1-DNA complex binds to the enzyme, much less fits into the active site for cleavage. The most likely explanation is that Tdp1 somehow promotes sufficient unfolding of the Tdp1-DNA complex for its active site to access the phosphoamide bond to be cleaved.

Patients who are affected by the SCAN1 disease which is caused by a mutation that replaces the active site His493 with arginine develop neurodegenerative symptoms during their teenage years (25). Contrary to earlier suggestions that this mutant protein is inactive (12,25,26), we showed recently that the Tdp1 H493R enzyme retains substantial enzymatic activity, but the mutation causes the accumulation of the covalent reaction intermediate (15). The covalent complex is long lived with a half-life of ~13 min. Based on these and other data we suggested that the accumulation of covalently bound mutant Tdp1 on the DNA likely contributes to the phenotype of the SCAN1 disease (15). If this were indeed the case, heterozygous carriers of the mutation would be expected to encounter the same problem as SCAN1 patients who carry the mutation in both alleles of *TDP1*. However the disease is completely recessive and

heterozygous carriers remain symptom free (25). Our observation here that wt Tdp1 can cleave the phosphoamide bond in an intact Tdp1-DNA covalent intermediate provides an explanation for how any H493R covalent complexes that accumulate in heterozygous individuals are repaired and likely accounts for the recessive nature of the SCAN1 defect.

The discovery that the DNA in the DNA-Tdp1 intermediate can be rapidly transferred from one Tdp1 molecule to another prompted us to reevaluate the previously-determined half life of the covalent complex. The half-life was originally determined to be ~13 min under conditions of a large excess of Tdp1 H493R over substrate. It is unknown what the conditions in a living cell are, but it is likely that the concentration of Tdp1 enzyme at a given site of DNA damage is lower than in our experimental conditions. Therefore it was of interest to determine whether an excess of enzyme had any effect on the half-life observed for the DNA-Tdp1 H493R covalent complex. Using a biotin-streptavidin-immobilized substrate to create conditions in which no free Tdp1 enzyme was available to potentially act on the covalent complex the half-life was determined to be ~16 min which is very close to the previously determined half-life of ~13 min. The fact that the half-life is slightly longer might be attributed to the fact that the immobilized substrate was duplex DNA, whereas the DNA was single-stranded in the original experiment. Thus, the half-life of the H493R covalent complex is independent of the amount of free enzyme present in the reaction and is likely to be similar in a living cell.

The only known naturally occurring substrate for Tdp1 other than topoisomerase I-DNA complexes are 3′ phosphoglycolate adducts (26,30). This observation suggested that Tdp1 might have additional roles in human cells besides the removal of stalled topoisomerase I-DNA complexes. Our data demonstrating that Tdp1 can act on a variety of naturally occurring substrates such as DNA, RNA, and terminal abasic sites is consistent with this hypothesis. Human Tdp1 always cleaves at the most 3′ backbone phosphate to remove one nucleoside from either DNA or RNA and leave a 3′ phosphate terminus. The yeast enzyme was reported to be unable to cleave the DNA phosphate backbone, but it is possible that the assay used was not sensitive enough to detect this activity (16). Since human Tdp1 does not possess a 3′ phosphatase activity, the exonuclease activity ends after the removal of one nucleoside. Yet, in combination with PNK which has 3′ phosphatase activity, Tdp1 could, in principle, become a processive 3′ exonuclease. Interestingly, Tdp1 has been shown to exist in complex with PNK in human cells (12,42).

Notably, Tdp1 always attacks the 3′-most phosphate in all of the substrates that were tested, including those containing 3′ biotin or a 3′ abasic site. Since the DNA-peptide substrate appears bent when bound in the active site (23), steric hindrance combined with limited flexibility of the bound DNA substrate likely prevents internal cleavage on such substrates. Interestingly, two other members of the PLD superfamily possess endonuclease activity (43,44). The three-dimensional crystal structure of bacterial endonuclease Nuc has been solved and shows that while the overall structures of human Tdp1 and Nuc are very similar, the substrate binding areas differ substantially (45,46).

It is worth noting that we have not yet encountered a 3′ adduct that could not be removed by Tdp1. Even the artificial 3′ biotin substrate was cleaved. Since the actual biotin moiety in this substrate is connected to the 3′ end of the DNA via a long linker, Tdp1 could potentially be used remove a variety of tags that are connected to DNA via this type of linker.

Pharmacologically relevant 3′ adducts like those generated by chain terminating nucleoside analogs used for the treatment of HIV-1 are likely to be substrates for Tdp1. Analogous to the role suggested for Ape1 (47,48), Tdp1 could act to protect the nuclear DNA from the detrimental effects of the incorporation of nucleoside analogs in anti-viral therapy.

Since several thousand abasic sites are generated spontaneously every day in a living cell (49,50), it was of particular interest to find that Tdp1 can act on the 3′ tetrahydrofuran moiety which is an abasic site mimic. Oxidative damage at DNA ends (i.e. the termini of DNA single- or double-strand breaks) or intermediates in the base excision repair process may represent substrates for Tdp1 *in vivo*. For example, it is conceivable that Tdp1 acts on the 3′ phospho α, β unsaturated aldehyde (3′ dRP) that results from β-elimination by the base-specific mammalian DNA glycosylases/AP lyases (for example, OGG1 or NTH1) (49,51-53). In this case Tdp1 would be an ideal candidate to remove the remaining sugar moiety from the DNA and allow repair to continue. Since the nervous system sustains more oxidative damage than most other cell types (54,55), it is conceivable that a direct role of Tdp1 in the repair of oxidative damage may explain why neurons are most affected in SCAN1 patients.

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REFERENCES

1. Yang SW, Burgin AB Jr. Huizenga BN, Robertson CA, Yao KC, Nash HA. Proc. Natl. Acad. Sci. U.S.A 1996;93:11534–11539. [PubMed: 8876170]
2. Wang JC. Annu. Rev. Biochem 1996;65:635–692. [PubMed: 8811192]
3. Wang JC. Nat Rev Mol Cell Biol 2002;3:430–440. [PubMed: 12042765]
4. Champoux JJ. Annu. Rev. Biochem 2001;70:369–413. [PubMed: 11395412]
5. Nitiss JL. Biochim. Biophys. Acta 1998;1400:63–81. [PubMed: 9748506]
6. Leppard JB, Champoux JJ. Chromosoma 2005;114:75–85. [PubMed: 15830206]
7. Porter SE, Champoux JJ. Nucleic Acids Res 1989;17:8521–8532. [PubMed: 2555774]
8. Pourquier P, Pommier Y. Adv Cancer Res 2001;80:189–216. [PubMed: 11034544]
9. Hsiang YH, Hertzberg R, Hecht S, Liu LF. J. Biol. Chem 1985;260:14873–14878. [PubMed: 2997227]
10. Hsiang YH, Liu LF. Cancer Res 1988;48:1722–1726. [PubMed: 2832051]
11. Barthelmes HU, Habermeyer M, Christensen MO, Mielke C, Interthal H, Pouliot JJ, Boege F, Marko D. J. Biol. Chem 2004;279:55618–55625. [PubMed: 15494395]
12. El-Khamisy SF, Saifi GM, Weinfeld M, Johansson F, Helleday T, Lupski JR, Caldecott KW. Nature 2005;434:108–113. [PubMed: 15744309]
13. Liu C, Pouliot JJ, Nash HA. DNA Repair (Amst) 2004;3:593–601. [PubMed: 15135727]
14. Nivens MC, Felder T, Galloway AH, Pena MM, Pouliot JJ, Spencer HT. Cancer Chemother Pharmacol 2004;53:107–115. [PubMed: 14605862]
15. Interthal H, Chen HJ, Kehl-Fie TE, Zotzmann J, Leppard JB, Champoux JJ. EMBO J 2005;24:2224–2233. [PubMed: 15920477]
16. Pouliot JJ, Robertson CA, Nash HA. Genes Cells 2001;6:677–687. [PubMed: 11532027]
17. Interthal H, Pouliot JJ, Champoux JJ. Proc. Natl. Acad. Sci. U.S.A 2001;98:12009–12014. [PubMed: 11572945]
18. Koonin EV. Trends Biochem Sci 1996;21:242–243. [PubMed: 8755242]
19. Ponting CP, Kerr ID. Protein Sci 1996;5:914–922. [PubMed: 8732763]
20. Morris AJ, Engebrecht J, Frohman MA. Trends Pharmacol Sci 1996;17:182–185. [PubMed: 8669123]
21. Exton JH. Rev Physiol Biochem Pharmacol 2002;144:1–94. [PubMed: 11987824]
22. Davies DR, Interthal H, Champoux JJ, Hol WG. J. Mol. Biol 2002;324:917–932. [PubMed: 12470949]
23. Davies DR, Interthal H, Champoux JJ, Hol WG. Chem Biol 2003;10:139–147. [PubMed: 12618186]
24. Raymond AC, Rideout MC, Staker B, Hjerrild K, Burgin AB Jr. J. Mol. Biol 2004;338:895–906. [PubMed: 15111055]
25. Takashima H, Boerkoel CF, John J, Saifi GM, Salih MA, Armstrong D, Mao Y, Quiocho FA, Roa BB, Nakagawa M, Stockton DW, Lupski JR. Nat Genet 2002;32:267–272. [PubMed: 12244316]

26. Zhou T, Lee JW, Tatavarthi H, Lupski JR, Valerie K, Povirk LF. Nucleic Acids Res 2005;33:289–297. [PubMed: 15647511]
27. Debethune L, Kohlhagen G, Grandas A, Pommier Y. Nucleic Acids Res 2002;30:1198–1204. [PubMed: 11861912]
28. Desai SD, Liu LF, Vazquez-Abad D, D′Arpa P. J. Biol. Chem 1997;272:24159–24164. [PubMed: 9305865]
29. Desai SD, Zhang H, Rodriguez-Bauman A, Yang JM, Wu X, Gounder MK, Rubin EH, Liu LF. Mol. Cell. Biol 2003;23:2341–2350. [PubMed: 12640119]
30. Inamdar KV, Pouliot JJ, Zhou T, Lees-Miller SP, Rasouli-Nia A, Povirk LF. J. Biol. Chem 2002;277:27162–27168. [PubMed: 12023295]
31. Povirk LF. Mutat. Res 1996;355:71–89. [PubMed: 8781578]
32. Rideout MC, Raymond AC, Burgin AB Jr. Nucleic Acids Res 2004;32:4657–4664. [PubMed: 15333697]
33. Davies DR, Interthal H, Champoux JJ, Hol WG. J. Med. Chem 2004;47:829–837. [PubMed: 14761185]
34. Burgin AB Jr. Huizenga BN, Nash HA. Nucleic Acids Res 1995;23:2973–2979. [PubMed: 7659520]
35. Schultz SJ, Zhang M, Champoux JJ. J. Mol. Biol 2004;344:635–652. [PubMed: 15533434]
36. Klumpp S, Krieglstein J. Eur J Biochem 2002;269:1067–1071. [PubMed: 11856347]
37. Gottlin EB, Rudolph AE, Zhao Y, Matthews HR, Dixon JE. Proc. Natl. Acad. Sci. U.S.A 1998;95:9202–9207. [PubMed: 9689058]
38. Rudolph AE, Stuckey JA, Zhao Y, Matthews HR, Patton WA, Moss J, Dixon JE. J. Biol. Chem 1999;274:11824–11831. [PubMed: 10207000]
39. Stewart L, Redinbo MR, Qiu X, Hol WG, Champoux JJ. Science 1998;279:1534–1541. [PubMed: 9488652]
40. Redinbo MR, Stewart L, Kuhn P, Champoux JJ, Hol WG. Science 1998;279:1504–1513. [PubMed: 9488644]
41. Oh MK, Yang H, Roberts MF. Biochim. Biophys. Acta 2003;1649:146–153. [PubMed: 12878033]
42. Plo I, Liao ZY, Barcelo JM, Kohlhagen G, Caldecott KW, Weinfeld M, Pommier Y. DNA Repair (Amst) 2003;2:1087–1100. [PubMed: 13679147]
43. Sapranauskas R, Sasnauskas G, Lagunavicius A, Vilkaitis G, Lubys A, Siksnys V. J. Biol. Chem 2000;275:30878–30885. [PubMed: 10880511]
44. Zhao Y, Stuckey JA, Lohse DL, Dixon JE. Protein Sci 1997;6:2655–2658. [PubMed: 9416618]
45. Stuckey JA, Dixon JE. Nat Struct Biol 1999;6:278–284. [PubMed: 10074947]
46. Davies DR, Interthal H, Champoux JJ, Hol WG. Structure (Camb) 2002;10:237–248. [PubMed: 11839309]
47. Jiricny J. Nature 2002;415:593–594. [PubMed: 11832924]
48. Chou KM, Cheng YC. Nature 2002;415:655–659. [PubMed: 11832948]
49. Barnes DE, Lindahl T. Annu Rev Genet 2004;38:445–476. [PubMed: 15568983]
50. Lindahl T, Wood RD. Science 1999;286:1897–1905. [PubMed: 10583946]
51. Ikeda S, Biswas T, Roy R, Izumi T, Boldogh I, Kurosky A, Sarker AH, Seki S, Mitra S. J. Biol. Chem 1998;273:21585–21593. [PubMed: 9705289]
52. Radicella JP, Dherin C, Desmaze C, Fox MS, Boiteux S. Proc. Natl. Acad. Sci. U.S.A 1997;94:8010–8015. [PubMed: 9223305]
53. Wiederhold L, Leppard JB, Kedar P, Karimi-Busheri F, Rasouli-Nia A, Weinfeld M, Tomkinson AE, Izumi T, Prasad R, Wilson SH, Mitra S, Hazra TK. Mol Cell 2004;15:209–220. [PubMed: 15260972]
54. Floyd RA. Proc Soc Exp Biol Med 1999;222:236–245. [PubMed: 10601882]
55. Poon HF, Calabrese V, Scapagnini G, Butterfield DA. Clin Geriatr Med 2004;20:329–359. [PubMed: 15182885]

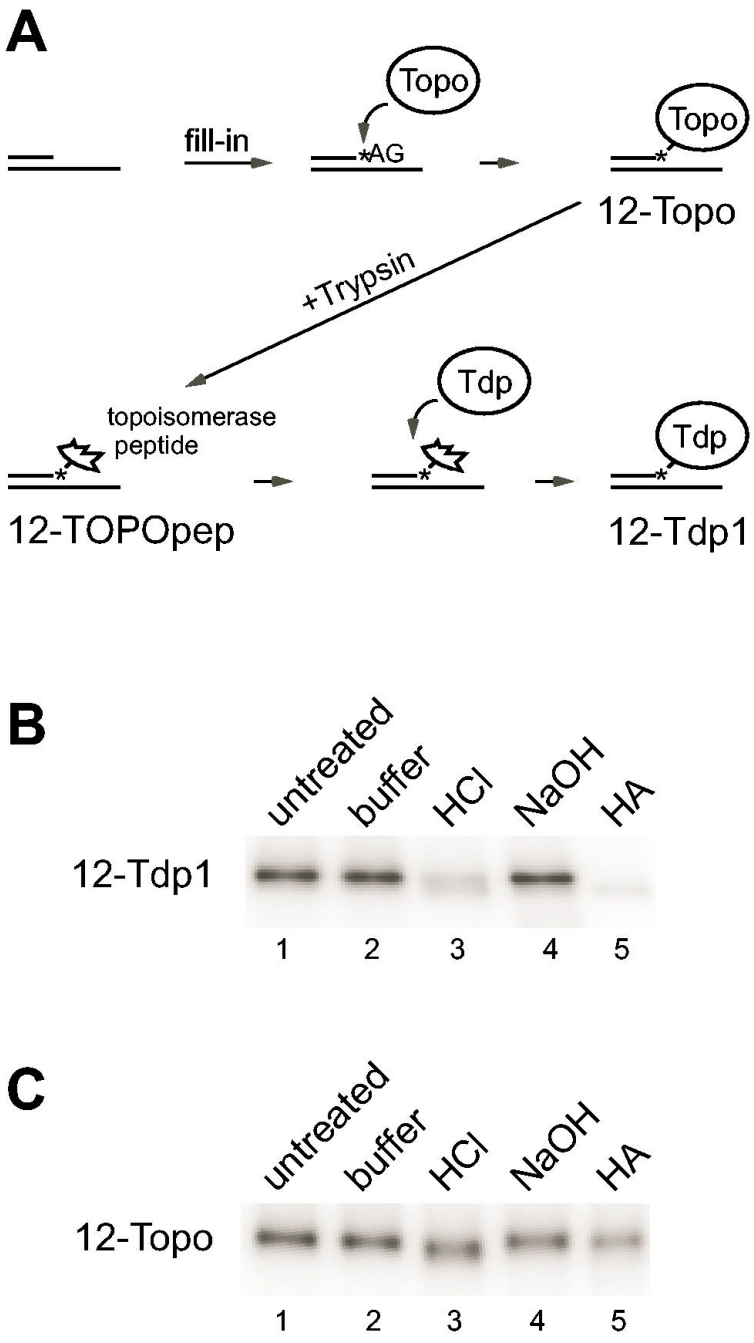


Fig. 1.

Chemical characterization of the phosphoamino acid bond in the Tdp1-DNA covalent intermediate. A. Schematic representation of substrate preparation. B. SDS-PAGE of Tdp1 H493R-DNA covalent reaction intermediate (12-Tdp1) samples treated under the indicated conditions. C. SDS-PAGE of treated topoisomerase I-DNA covalent complexes (12-Topo).

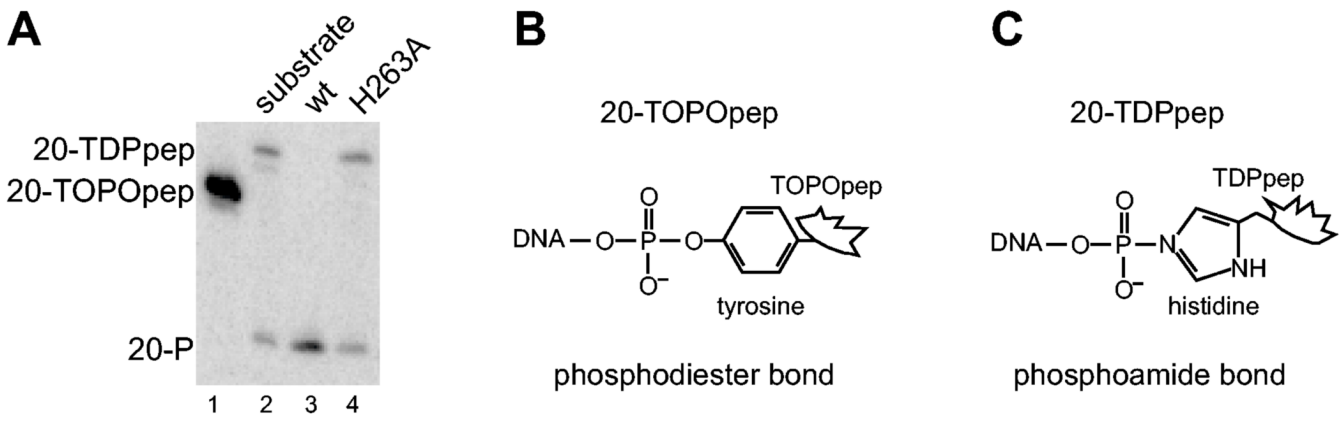


Fig. 2.

Tdp1 activity assay on the phosphoamide bond between a DNA 3′ phosphate and a Tdp1 tryptic peptide. A. 20-TDPpep (lane 2) was generated by incubating the H493R mutant protein with 20-TOPOpep (lane 1) followed by trypsin digestion. 20-TDPpep was incubated with wt Tdp1 (lane 3) or Tdp1 H263A (lane 4) followed by sequencing gel analysis. B. Structure of the DNA-topoisomerase I tryptic peptide complex in which the topoisomerase I fragment (TOPOpep) is linked to the DNA 3′ phosphate through a phosphodiester bond with the active site tyrosine. C. Structure of the DNA-Tdp1 tryptic peptide complex. The Tdp1 peptide (TDPpep) is connected to the DNA via a phosphoamide bond between the nucleophilic His263 and the 3′ DNA phosphate.

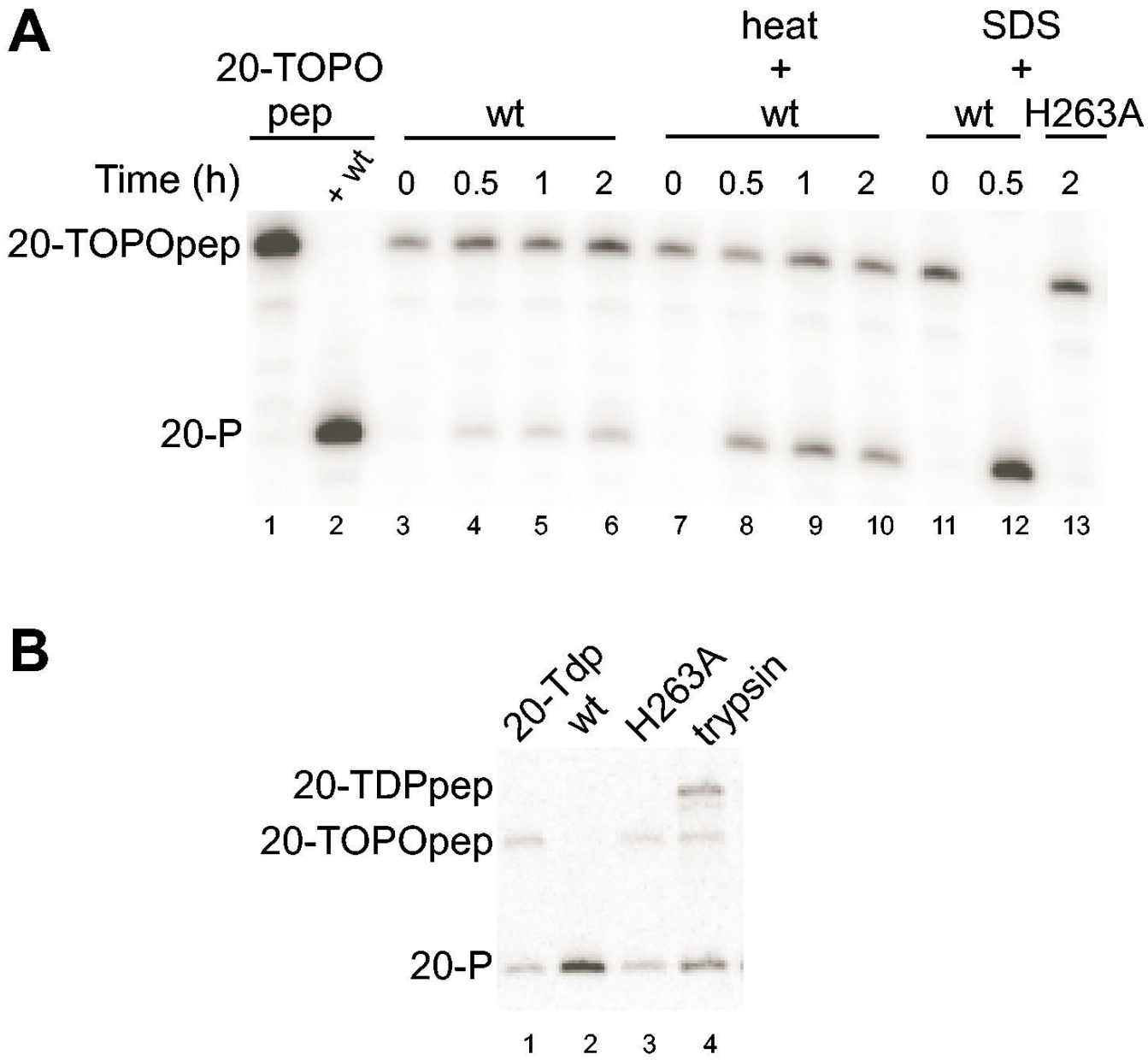


Fig. 3.

Tdp1 activity on topoisomerase I-DNA and Tdp1-DNA covalent complexes. A. Tdp1 activity assays on topo70-DNA covalent complexes that were either untreated (lanes 3-6), incubated at 65°C for 10 min (lanes 7-10), or SDS denatured (lanes 11-13) before Tdp1 addition. At the indicated times samples were taken, the reactions stopped with SDS, and trypsin digested before sequencing gel analysis. In lane 1, 20-TOPOpep was loaded as a size marker for the 20-mer DNA with a tryptic topoisomerase I peptide. Lane 2 contains the product of the reaction of wt Tdp1 on 20-TOPOpep, a 20-mer with a 3′ phosphate group (20-P). B. Tdp1 activity assay on SDS denatured Tdp1-DNA complexes. 20-TOPOpep was incubated with Tdp1 H493R and the reaction stopped with SDS (lane 1). To visualize the covalent Tdp1-DNA intermediate, a portion of the reaction was digested with trypsin before loading (lane 4). Wt (lane 2) or H263A

(lane 3) Tdp1 were added to the substrate mix shown in lane 1 and the reactions were analyzed on a sequencing gel.

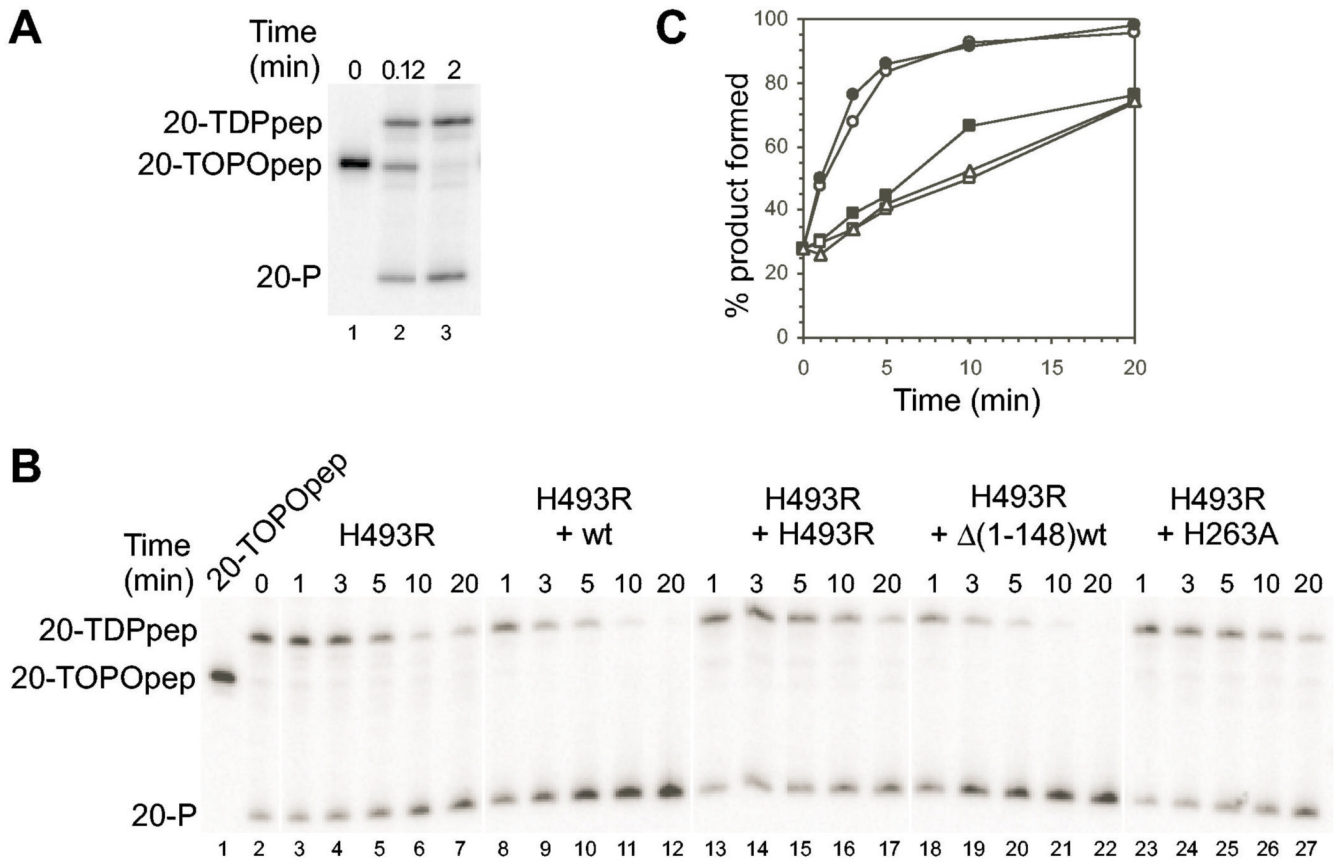


Fig. 4.

Activities of wt and Tdp1 mutants on the H493R-DNA reaction intermediate. A. Tdp1 H493R and 20-TOPOpep were incubated for the indicated times, treated with trypsin and analyzed on a sequencing gel. B. H493R was incubated with 20-TOPOpep (lane 1) for 2 min (lane 2) which serves as the zero time point for the subsequent reactions. At this time point , the indicated second enzymes (lanes 8-27) were added to the reaction mixtures and samples were taken at the time points shown. No second enzyme was added to the time course experiment shown in lanes 3-7. All samples were digested with trypsin, and analyzed on a sequencing gel. C. The accumulation of the reaction product 20-P over time is plotted for the reactions shown in B. H493R alone (filled squares), plus wt (open circles), plus additional H493R (open squares), plus Δ(1-148) wt (filled circles), and plus H263A (open triangles).

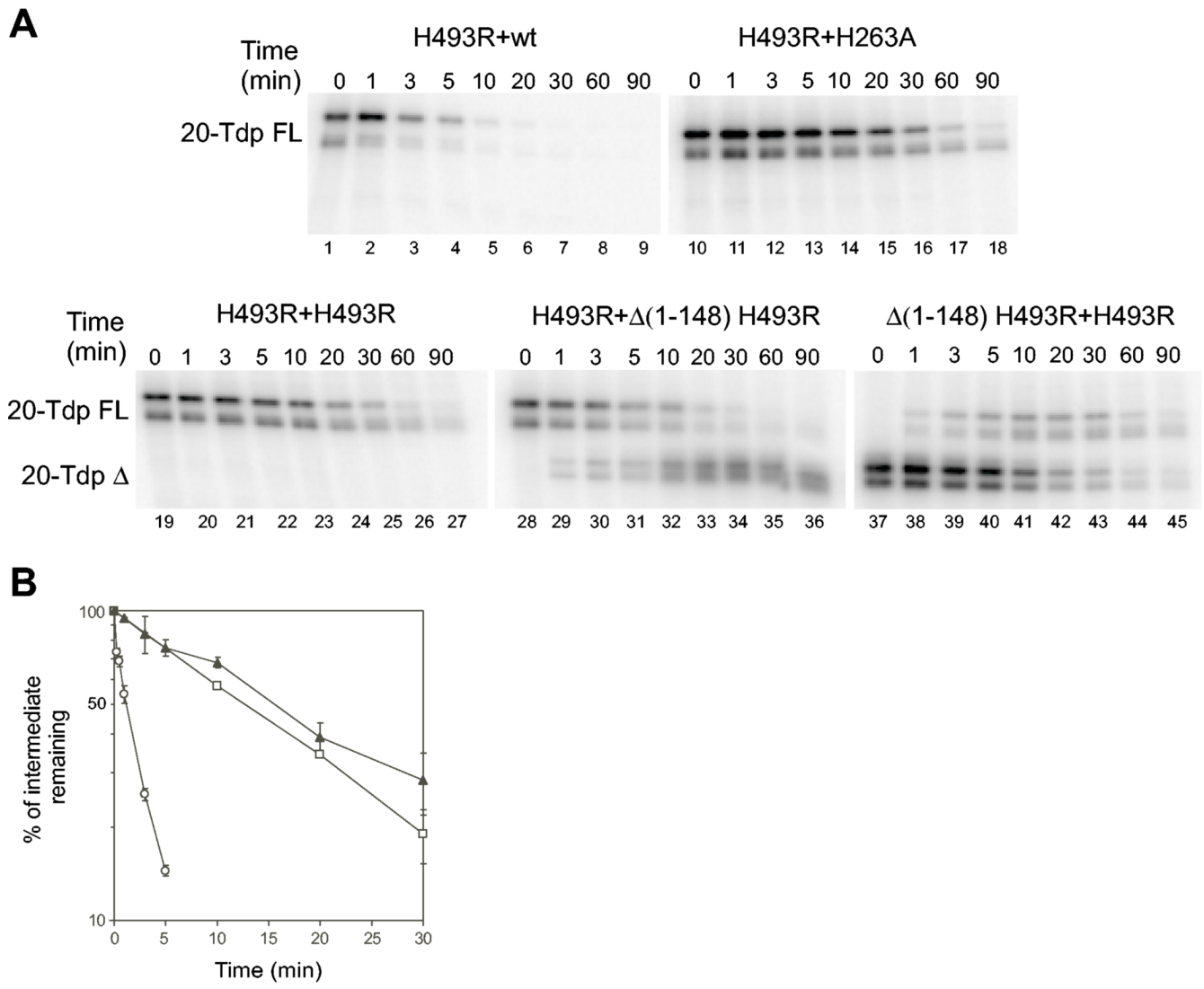
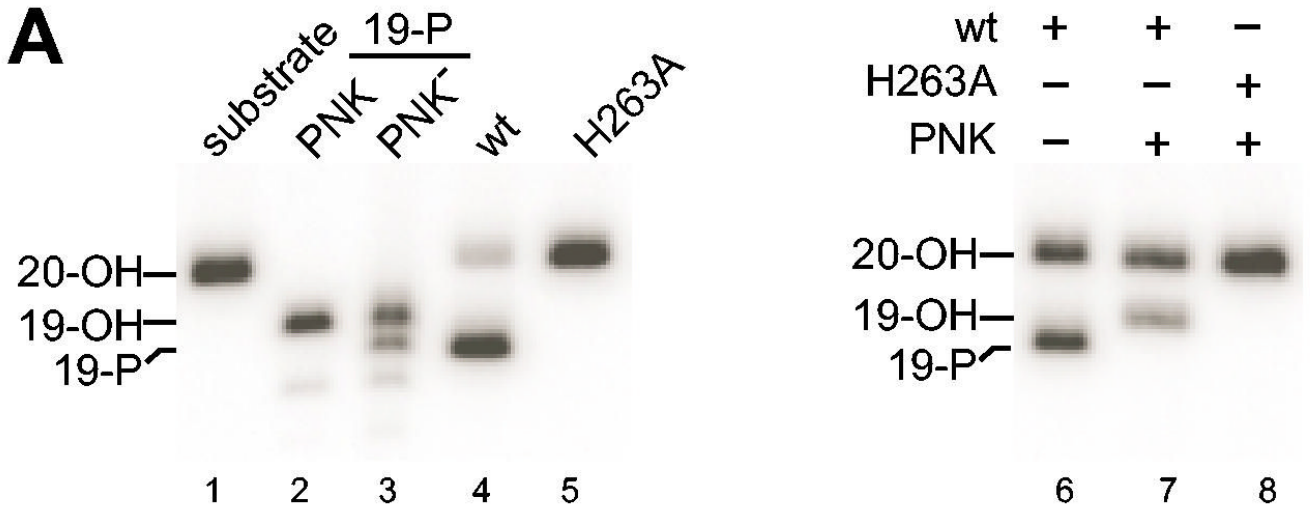


Fig. 5.

The DNA in the covalent reaction intermediate is "handed-off" from one Tdp1 molecule to the other. A. Either full length H493R or N-terminally truncated Δ(1-148) H493R was incubated with 20-TOPOpep for 2 min to form 20-Tdp FL and 20-Tdp Δ, respectively. The indicated second enzyme was added to the reactions at 2 min and referred to as the zero time point for the subsequent time course analyses. Samples were taken at the time points shown and analyzed by SDS-PAGE. B. The decay of the Tdp1-DNA intermediate over time as determined from a parallel sequencing gel analysis is plotted for a subset of the time course experiments shown in (A). Averages of three experiments are shown with error bars representing standard deviations. Kinetics of decay are shown for reactions containing additional H493R (open squares), and after the addition of wt Tdp1 (open circles)). The decay of the double-stranded DNA-Tdp1 H493R covalent complex in the absence of excess free Tdp1 H493R was plotted over time (filled triangles).



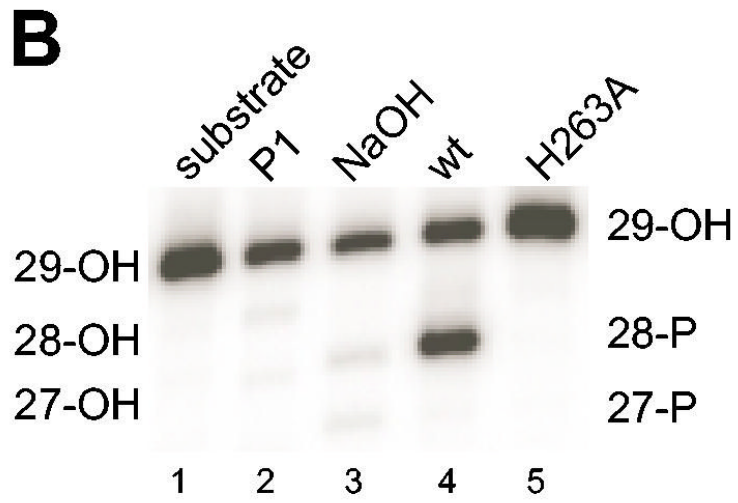


Fig. 6.

Tdp1 activity assays on DNA and RNA substrates. A. A 20-mer DNA oligonucleotide with a 3′ hydroxyl (lane 1) was incubated with either wt (lane 4) or H263A (lane 5) Tdp1. A 19-mer with a 3′ phosphate was 5′ end-labeled with either wt PNK (lane 2) or a 3′ phosphatase minus mutant PNK (PNK^{-}, lane 3) to obtain size markers for a 19-mer with a 3′ hydroxyl (19-OH) and a 19-mer with a 3′ phosphate group (19-P), respectively. For the samples loaded in lanes 6-8, the 20-mer DNA was first incubated with the indicated Tdp1 variant and then treated with PNK (lanes 7 and 8) or left untreated (lane 6). The samples were analyzed on a sequencing gel. B. A 29-mer RNA oligonucleotide annealed to a 48-mer DNA nucleotide (lane 1) was incubated with either wt Tdp1 (lane 4) or Tdp1 H263A (lane 5) before sequencing gel analysis. The RNA ladders with either 3′ hydroxyl or 3′ phosphate groups were generated by incubating the 29-mer RNA with P1 nuclease (lane 2) or NaOH (lane 3), respectively.

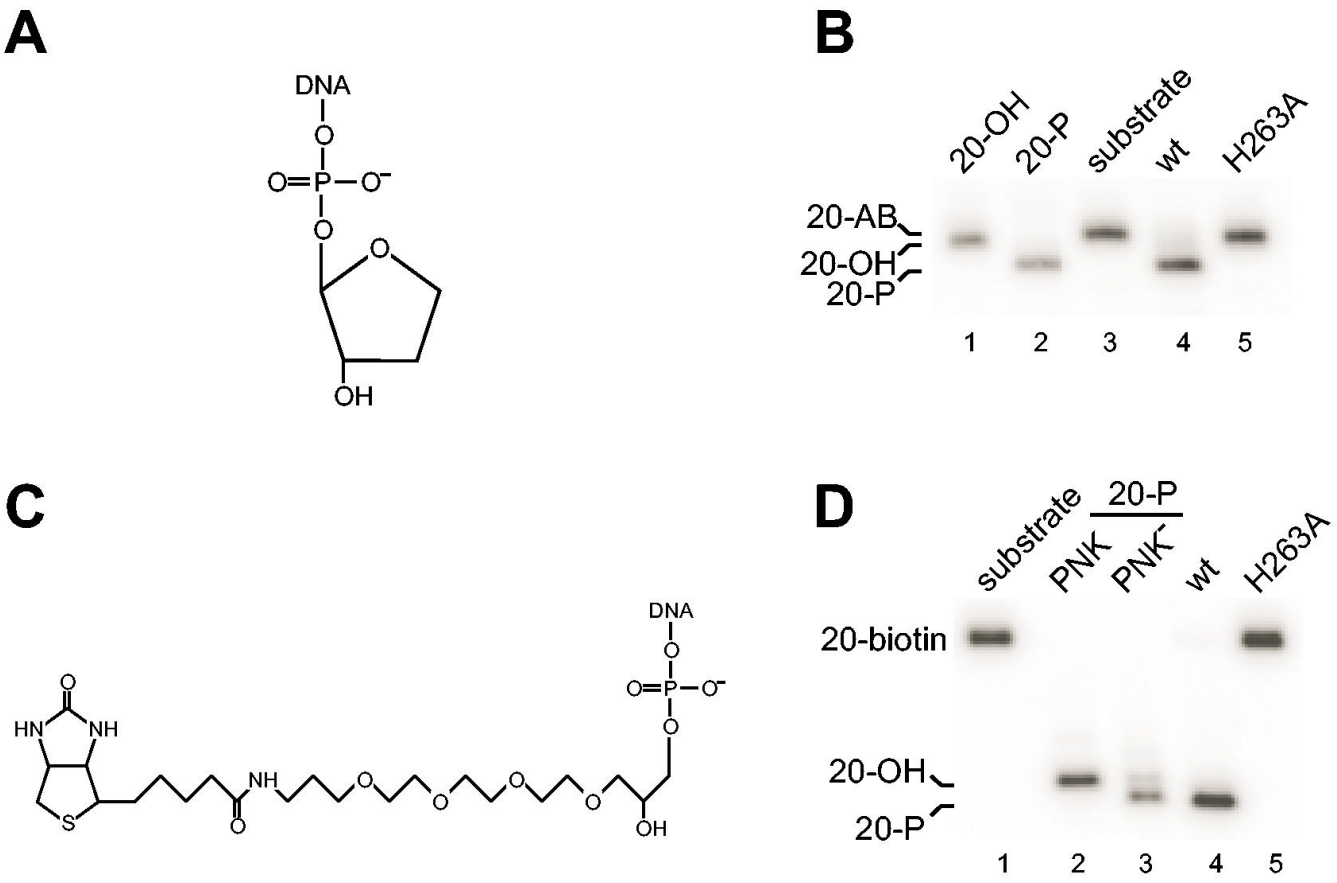


Fig. 7.

Tdp1 activity assays on DNA oligonucleotides with 3′ tetrahydrofuran or 3′ biotin moieties. A. Structure of the abasic site mimic tetrahydrofuran on the DNA 3′ end. B. A 20-mer oligonucleotide containing the tetrahydrofuran moiety on the 3′ end (lane 3, 20-AB) was incubated with wt Tdp1 (lane 4) or the H263A mutant enzyme (lane 5). A corresponding 20-mer with a 3′ hydroxyl group (lane 1, 20-OH) was used as a size marker. The 20-mer with a 3′ phosphate (20-P) was generated by incubating a 20-mer with a 3′ tyrosyl moiety with wt Tdp1 (lane 2). C. Structure of biotin connected to the 3′ end of the DNA by a 15-atom linker. D. Wt Tdp1 (lane 4) or H263A (lane 5) were incubated with a 3′ biotinylated 20-mer DNA (lane 1, 20-biotin) and analyzed on a sequencing gel. To generate size markers (20-OH and 20-P),` a corresponding 3′ phosphorylated 20-mer was labeled with either wt PNK (lane 2) or a 3′ phosphatase minus mutant PNK (PNK^{-}) (lane 3).