Substrate Specificity of Tyrosyl-DNA Phosphodiesterase I (Tdp1)*

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Tyrosyl-DNA phosphodiesterase I (Tdp1) hydrolyzes 3′-phosphotyrosyl bonds to generate 3′-phosphate DNA and tyrosine in vitro. Tdp1 is involved in the repair of DNA lesions created by topoisomerase I, although the in vivo substrate is not known. Here we study the kinetic and binding properties of human Tdp1 (hTdp1) to identify appropriate 3′-phosphotyrosyl DNA substrates. Genetic studies argue that Tdp1 is involved in double and single strand break repair pathways; however, x-ray crystal structures suggest that Tdp1 can only bind single strand DNA. Separate kinetic and binding experiments show that hTdp1 has a preference for single-stranded and blunt-ended duplex substrates over nicked and tangled substrate DNA conformations. Based on these results, we present a new model to explain Tdp1/DNA binding properties. These results suggest that Tdp1 only acts upon double strand breaks in vivo, and the roles of Tdp1 in yeast and mammalian cells are discussed.

DNA topoisomerases are ubiquitous enzymes that catalyze changes in DNA topology by altering the linkage of DNA strands (1). Eukaryotic topoisomerase I (TopI) uses an active site tyrosine residue to cleave one strand of DNA, forming a 3′-phosphotyrosine intermediate. This opening of the DNA backbone is necessary to allow the removal of superhelical tension that is generated during replication and transcription. The phosphodiester DNA backbone is restored when the 5′-hydroxyl, generated during cleavage, attacks the 3′-phosphotyrosyl phosphodiester (2). Because the rate of religation is normally much faster than the rate of cleavage, the steady state concentration of topoisomerase-DNA adducts is extremely low. This is important to maintain the integrity of the genome. However, TopI-DNA adducts can accumulate in the presence of naturally occurring DNA damage such as nicks (3), abasic sites (4), modified bases (5), or modified sugars (6) or as a result of exposure to a variety of chemotherapeutic drugs such as camptothecin (7, 8). These lesions can be converted to more permanent single strand breaks (SSBs) or double strand breaks (DSBs) via the action of RNA transcription and DNA replication machinery.

Tyrosyl-DNA phosphodiesterase (Tdp1) catalyzes the hydrolysis of the phosphodiester bonds between the 3′-end of DNA and a tyrosine residue in vitro (9). In yeast, Tdp1 has been implicated in the repair of TopI-DNA adducts via the double strand break repair (DSBR) pathway, although it is also clear that additional Tdp1-independent repair pathways can correct TopI-mediated lesions (10, 11). The role of Tdp1 in mammalian cells is not as well understood. It has been proposed that Tdp1 participates in the single strand break repair (SSBR) pathway (12, 13). Understanding the biological role of Tdp1 is important since mutations that are known to inactivate Tdp1 (14) are linked to the genetic disease spinocerebellar ataxia with axonal neuropathy (15). In addition, overexpression of Tdp1 in human cells confers resistance to the well known anticancer agent camptothecin (16). Although this same phenotype is not observed in yeast cells, this result suggests that Tdp1 may be a new molecular target for novel anticancer drugs (17).

Initial biochemical studies showed that yeast Tdp1 catalyzed the hydrolysis of the phosphodiester bonds between the 3′-end of DNA and a tyrosine residue in vitro with nanomolar affinity ($K_m = 10$ nM); 5′-phosphotyrosyl bonds were not cleaved by Tdp1 (9). Early studies showed that yeast Tdp1 prefers single-stranded substrates versus DNA substrates containing a 3′-phosphotyrosine at a nick within double-stranded DNA (9). Subsequent studies have focused primarily on the protein requirements for Tdp1 activity on DNA-protein substrates. For example, single strand oligonucleotides containing large peptide fragments (>11 amino acids) are hydrolyzed very slowly, and it is presumed that in vivo, the topoisomerase is proteolyzed to one (tyrosine) or a few amino acids before it is ultimately acted upon by Tdp1 (9, 18). In addition, single-stranded oligonucleotides containing a 3′-tyrosine residue, 3′-(4-nitro)phenyl or 3′-(4-methyl)phenyl phosphate, are very efficient substrates in vitro (14). Finally, Tdp1 has also been shown to cleave 3′-phosphoglycolate linkages (19), emphasizing the general lack of specificity for the 3′-leaving group. Tdp1 may be involved in repairing a variety of different DNA lesions through hydrolysis of a 3′-phosphodiester.

Tdp1 may also have multiple roles in the cell. For example in Drosophila, Tdp1 (glaikit; gkt) is essential for the formation of epithelial polarity and for neuronal development during embryogenesis, and it has been proposed that Tdp1 is involved in the localization of membrane proteins (20). Although no substrate or mechanism has been proposed to support this function, Tdp1 is a member of the phospholipase D (PLD) superfamily of enzymes (21), and other members of this superfamily (PLD1) participate in membrane vesicle trafficking by hydrolyzing phosphatidylcholine to generate phosphatidic acid (22–24). These results emphasize the importance of understanding the in vitro binding properties and

* This study was supported in part by a Cooperative Planning of the SDSU/UCSD Cancer Partnership Grant CCA92079-01A2 (to A. C. R.) and by NIGMS Grant GM55896 (to A. B. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This paper is available on line at http://www.jbc.org

** Supported by Predoctoral Fellowship 1F31 GM66372-01 from the National Institutes of Health.

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‡ The abbreviations used are: TopI, topoisomerase I; Tdp1, tyrosyl-DNA phosphodiesterase 1; hTdp1, human Tdp1; DB, double strand break; SSB, single strand break; DBSR, double strand break repair; SSBR, single strand break repair; PLD1, phospholipase D superfamily; 6-FAM, (3′-6′-dipivaloylfluoresceinyl)-6-carboxamidomethylxyl.
catalytic mechanism of Tdp1 to better understand its biological role(s).

Tdp1 can cleave 3’-tyrosine or 3’-(4-nitro)phenyl mononucleotides (9, 25), but these substrates are relatively poor (14). The preference for oligonucleotide substrates versus mononucleotide substrates is expected since crystal structures show that 3 conserved residues in Tdp1 make specific hydrogen bond contacts to two phosphodiester bonds upstream of the cleavage site (26). Based on the crystal structure of human Tdp1 (27), it was proposed that the DNA portion of the substrate bound within a positively charged cleft on the enzyme surface. This hypothesis was partially confirmed when crystal structures of Tdp1 bound to a small oligonucleotide were obtained (26, 28, 29). In these structures, 3 or 4 nucleotides of a 6-mer single strand oligonucleotide could be visualized near the proposed binding cleft in the electron density maps. Surprisingly, the cleft is too narrow to accommodate duplex DNA, and it was proposed that Tdp1 binds only single-stranded DNA. The ability of human Tdp1 to bind and cleave single-stranded, double-stranded, and other structures that could be encountered in vitro is not known. In this study, we determine the kinetic and binding properties of Tdp1 for DNA substrates that may arise at different stages of topoisomerase I-mediated damage. The results show that human Tdp1 has a preference for single-stranded or blunt-ended duplex oligonucleotides. We present a new binding model to explain how Tdp1 can bind and cleave both single-stranded and double-stranded duplexes. The results are consistent with genetic data indicating that Tdp1 could function in DSBR and SSBR after the topoisomerase I-induced damage is converted to a double strand break.

MATERIALS AND METHODS

Preparation of hTdp1 and DNA Substrates—The human Tdp1 1–148 was prepared as described previously (14). The 3’-phosphorysryosine oligonucleotide (5’-CGTTGAAGCCGCTTTT-3’, where Y = tyrosine) and 3’-phosphorysryosine oligonucleotide 5’-(6-FAM) oligonucleotides (5’-XCGTTGAAGCCGCTTTT-3’, where Y = tyrosine and X = (3’-dipivaloylfluoresceinyl)-6-carboxamidohexyl) were purchased from Midland Certified Reagent Co. (Midland, TX). The 6-FAM-donating phosphoramidite was purchased by Midland Certified Reagent Co. from Glen Research (Sterling, VA). For gel-based kinetic assays, the 3’-phosphorysryosine oligonucleotide was 5’-radiolabeled with [32P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) at 37 °C for 15 min followed by 10 min at 90 °C to inactivate the kinase. Radiolabeled 3’-phosphorysryosine oligonucleotide was hybridized to: an equivolament of 5’-AAAGCGAGGCCTACAGC-3’ to make blunt duplex substrate; or a 2-fold molar excess of 5’-GCAATCCGCGGACTTCAAGACATCCTGGACAC-3’ to make tailed duplex substrate; or a 2-fold molar excess of 5’-GCAATCCCTGGAGACAGGCTTCAATC-3’ to make nicked duplex substrate. For mismatch duplex substrates, the 3’-phosphorysryosine oligonucleotide was hybridized to: a 5’-AAAGCGAGGCCTACAGC-3’ or an equivolament of 5’-GCTGGAGCTGCTGATAC-3’ to make nicked triple strand substrate. For mismatch duplex substrates, the 3’-phosphorysryosine oligonucleotide was hybridized to: either a 10-fold molar excess of 5’-GCAATCCGCGGACTTCAAGACATCCTGGACAC-3’ or a 15-fold molar excess of 5’-GCAATCCCTGGAGAGGCTTACAGC-3’. Hybridizations were performed in 50 mM Tris-HCl (pH 6.5), 5 mM MgCl2, 80 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, and 40 μg/ml bovine serum albumin. Enzyme stocks were diluted in 50 mM Tris-HCl (pH 6.5), 100 mM NaCl, 5 mM dithiothreitol, 10% glycerol, 500 μg/ml bovine serum albumin. Kinetic analysis with single strand or blunt end (0, 1, or 2 mismatches) duplex substrates contained 50 μM hTdp1; kinetic analysis with double strand nicked or tailed substrates contained 200 μM hTdp1.

Unless otherwise noted, all reactions were performed at 37 °C in 96-well v-bottom reaction plates, quenched by an equal volume of 8 μM urea, 0.05% SDS, 30% glycerol, and 0.25% bromphenol blue, and then resolved on 20% acrylamide sequencing gels. The concentration of 3’-phosphate DNA was determined by measuring the fraction of substrate converted to product by densitometry analysis of the gel image. Initial velocities were determined by plotting the concentration of 3’-phosphate DNA as a function of time. Only concentrations representing less than 20% of initial substrate concentrations were used, all lines were extrapolated to zero product at the start of the reaction, and at least five time points were used to determine the slope of the line (velocity). Finally, all velocity measurements were performed in triplicate on three different days. Apparent $K_{m}$ and $V_{max}$ values were determined by fitting the initial velocity versus substrate concentration to the Michaelis-Menten equation, $V = (V_{max} \cdot [S]) / (K_{m} + [S])$. The $k_{cat}$ was calculated by dividing the reaction velocity by the reaction enzyme concentration. Very similar values were obtained from linear regression analysis of Eadie-Hofstee plots of the same data (data not shown).

Fluorescence Polarization—Fluorescence polarization experiments were performed in a POLARstar Optima (BMG Labtechnologies, Durham, NC). Reactions were incubated at 37 °C for 60 min prior to measurement to allow the enzyme binding to reach equilibrium, as determined by the absence of change in the observed polarization values. Reactions were performed in triplicate in a black flat-bottom 96-well plate at 37 °C and contained 30–90 nM 5’-fluorescein substrate in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 5 mM MgCl2, 80 mM KCl, 2 mM dithiothreitol, and 40 μg/ml bovine serum albumin. Polarization was measured by excitation with vertically polarized light at a wavelength of 485 nm with four cycles of 100 flashes/cycle and subsequent measurement of fluorescence emission at a wavelength of 520 nm in the vertical and horizontal positions. The polarization data were fit using Kaleidagraph v.3.51 software to the equilibrium binding equation (39), $P_{obs} = (I_{P}) / (I_{P} + I_{P} \cdot (K_{d} \cdot ([DNA] + [hTdp1])))$. Where $P_{obs}$ is the observed polarization of the 5’-(6-FAM) oligonucleotide substrate at any hTdp1 concentration, $P_{max}$ is the maximum polarization value, $P_{min}$ is the minimum observed polarization value, and $K_{d}$ is the equilibrium dissociation constant.

Structure Modeling—An idealized B-form duplex double-stranded DNA was modeled onto the binding site of hTdp1 by aligning the first three sugar-phosphate backbones of the 24-mer with the first three sugar-phosphate backbones of the oligonucleotides from the crystal structures (1N0P and 1RHO). The 24-mer was positioned by hand using the program XFIT (40) and oriented to minimize protein contacts while maintaining the proximity of the first four phosphates of the sugar-phosphate backbone. The duplex was found to position into the binding site without any direct protein-DNA clashes. The model was then minimized by positional refinement using the program CNS (41). Minimization allowed minor changes in atomic movements to optimize binding. Minor changes were noted for residues Arg-232, Lys-231, and Trp-590.

RESULTS

Topoisomerase I-mediated DNA damage can occur during replication or transcription (11, 30), resulting in a stable 3’-topoisomerase I-DNA covalent adduct. This covalent adduct can be converted into a variety of potential DNA products including nicked, double strand tailed (would also mimic duplex with single strand gap), double strand blunt, or single-stranded DNA. These potential substrates are diagrammed in Fig. 1. It is not known at what stage of repair Tdp1 acts. To better understand the natural substrate(s) in vivo, we have synthesized and characterized the ability of Tdp1 to cleave these potential DNA substrates. In all studies, the substrates contained a single tyrosine residue since it is generally agreed that the protein portion of the covalent adduct is proteolyzed before being acted upon by Tdp1.

All of the reactions were performed with an N-terminal deletion of human Tdp1, Δ1–148 since all crystal structures of hTdp1 have been obtained using this N-terminal deletion, and we have independently confirmed previous studies (21, 27) demonstrating that full-length hTdp1 and hTdp1 Δ1–148 have indistinguishable specific activities in vitro (14). An alteration in the substrate binding site or catalytic efficiency would alter the specific activity of the enzyme. It has been proposed that
Tdp1 Substrate Specificity

Tdp1 does participate in a multiprotein repair complex in vivo (16, 31), and we cannot rule out the possibility that the N terminus of hTdp1 participates in protein-protein interactions. However, the N terminus of Tdp1 is extremely variable in size and amino acid identity among Tdp family members, which argues that this region is not essential for activity (21).

We previously reported that hTdp1 cleaves single strand oligonucleotides with an apparent second order rate constant of \(-1 \times 10^8 \text{M}^{-1}\text{s}^{-1}\) (14). This relatively large specificity constant suggests that the reaction is diffusion-limited under standard conditions. Under these conditions, the apparent \(K_m\) value may not reflect the true affinity for the substrate, and large differences in catalytic efficiency may not be detected. Because we wanted to determine how well the enzyme cleaves these different substrates, we first determined reaction conditions in which hTdp1 would not be diffusion-limited. Tdp1 uses a general acid base catalytic mechanism, and we reasoned that low-pH conditions and partial rate-limiting. Using sin-
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Fig. 2. Determination of non-diffusion-limited reaction condi-
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To monitor the cleavage reaction, only the 3'-phosphotyrosine oligonucleotide was 5'-end labeled. Hybridization conditions were optimized for each substrate and analyzed on native
gels to ensure that labeled oligonucleotide was fully hybridized and homogeneous. Before and after reaction with hTdp1, a portion of the reaction was resolved on native polyacrylamide gels to ensure that the DNA remained completely hybridized during the reaction (Fig. 3A). After the addition of hTdp1, aliquots were removed, quenched, and then resolved on a denaturing polyacrylamide sequencing gel. Enzyme activity was quantitated by determining the concentration of the faster-migrating 3'-phosphate DNA product as a function of time (Fig. 3B). Reaction velocities were determined as a function of substrate concentration (Fig. 3C) and used to determine \(k_{cat}\) and \(K_m\) values for each of the substrates (see “Materials and Methods”).

A summary of the kinetic data is presented in Table I. The apparent second order rate constants for single strand and
double strand blunt and tailed substrates are essentially identical
\((-5 \times 10^8 \text{M}^{-1}\text{s}^{-1})\); however, nicked and tailed substrates are cleaved very poorly by Tdp1. Both the turnover rate and the specificity constants are 10-fold lower for the nicked and tailed

![Fig. 3](image_url)

Fig. 3. Kinetic analysis of DNA substrates. In all experiments, only the DNA oligonucleotide containing the 3'-phosphotyrosine link-
age was radiolabeled. This oligonucleotide was hybridized to complement-
ary oligonucleotides as described under “Materials and Methods” to
generate double strand blunt, double strand tailed, and double strand nicked substrates. A, aliquots of each DNA substrate were
resolved on a non-denaturing polyacrylamide gel before (left lane) and
after (right lane) to ensure that the duplex substrates remained hybrid-
ized during the course of the reaction. The position of each substrate is
indicated. B, after addition of hTdp1, aliquots were removed, quenched,
and then resolved on a denaturing polyacrylamide sequencing gel. The
positions of the denatured 3'-phosphotyrosine (substrate) and 3'-phos-
phate (product) oligonucleotides are indicated. C, reaction velocities
were determined as a function of DNA substrate concentration, and the
observed rate of reaction (\(k_{obs} = \text{velocity}/[\text{enzyme}]\)) is plotted as a
function of substrate concentration. Reactions were performed on three
different days, and all three data points are plotted and fit to the
Michaelis-Menten equation (see “Materials and Methods”).

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duplex substrates. This result may be expected since crystal structures of a Tdp1-DNA-peptide complex assembled around the phosphodiester mimic vanadate show that several catalytic residues contact and stabilize the trigonal bipyramidal intermediate. Presumably, DNA on the complementary strand in the nicked and tailed substrates sterically hinders the ability of active residues to contact and stabilize the transition state.

The fact that blunt duplex DNA is cleaved slightly faster than single strand DNA was unexpected since the current DNA binding model proposes that only one strand of DNA can be accommodated by hTdp1 (28). One possible explanation (27) is that the DNA is first “melted” in the blunt end substrate, allowing access to the 5′-phosphotyrosyl bond. If this explanation is true, then placing DNA mismatches at the end of the blunt end duplex should facilitate melting and improve the ability of Tdp1 to cleave this substrate. We therefore constructed blunt end duplexes with 0, 1, or 2 mismatches in the substrate complement. To ensure that these substrates hybridized as designed, they were analyzed on a native polyacrylamide gel both before and after hTdp1 reaction (Fig. 4A). As can be seen in Fig. 4B, the number of mismatched bases at the substrate 3′-end is inversely correlated to the reaction efficiency. The turnover number decreases incrementally with each additional mismatch at the 3′-end of the phosphotyrosyl-containing oligonucleotide (Fig. 4C), demonstrating that mismatches on the substrate terminus not only fail to improve the reaction efficiency but in fact increasingly hinder the reaction. One possible explanation for this unexpected outcome is that the unpaired terminal region cannot be accommodated by the enzyme active site and provides steric hindrance. This result remains in conflict with the current substrate binding model for Tdp1 (28) but does support alternative binding models (see “Discussion”).

One possible complication in these studies is the fact that the crystal structures were obtained with a non-hydrolyzable phosphodiester mimic (vanadate) and therefore, provide more information about binding than catalysis. However, the kinetic studies above require both substrate binding and catalysis. We therefore sought to analyze the ability of hTdp1 to bind the four substrates diaphragm in Fig. 1. We first attempted gel mobility shift assays using radioactively labeled DNA molecules and a catalytically inactive enzyme; however, we were unable to detect protein-specific gel shift using this method. We therefore developed a fluorescence polarization method for measuring hTdp1 binding to DNA. For these experiments, the 3′-phosphotyrosine-containing oligonucleotide was 5′-end labeled with fluorescein. Hybridization of the substrates was optimized and monitored in separate reactions using radiolabeled complementary oligonucleotides (data not shown), and a catalytically inactive enzyme (H263A) was used to prevent hydrolysis of the substrate during the course of the experiment. Structural data indicates that histidine 263 is not involved in DNA binding but is poised for nucleophilic attack of the substrate 3′-phosphotyrosyl bond (26, 27), and we have previously shown that this mutant is completely devoid of any catalytic activity (14). In these experiments, fluorescence anisotropy was measured as a function of Tdp1 concentration. The anisotropic signal is proportional to the tumbling rate in solution, and therefore, a measure of the molecular weight (32). The results in Fig. 5A show that as Tdp1 concentration increases, the anisotropic signal also increases until the concentration of Tdp1 equals the concentration of DNA (i.e. all of the binding sites are occupied by Tdp1). This result confirms the assumption that one molecule of Tdp1 binds one molecule of DNA and allows the direct determination of a dissociation binding constant ($K_d$). To determine the relative affinities, the experiment was repeated with each of the potential DNA substrates (Fig. 1). The results in Fig. 5B show that Tdp1 binds single strand and blunt end duplex substrates with equal affinity ($K_d$ values of ~150 nM). In these experiments, Tdp1 bound the nicked and tailed substrates with such poor affinity that an absolute value of $K_d$ could not be determined because the anisotropic signal could not be clearly saturated at the highest protein concentrations. One possible explanation for these results is that Tdp1 preferentially binds small DNA molecules (single strand and blunt end duplexes). To control for this possibility, we also measured Tdp1-dependent binding to a single strand oligonucleotide of

### Table 1

| Tdp1 Substrate Specificity | $K_M$ (nM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_M$ | $K_D$ (nM) |
|---------------------------|------------|----------------------|---------------|------------|
| de blunt                 | 80±20      | 24±1                 | 5.0 $\times 10^8$ | 150±25     |
| MM2                      | 140±50     | 47±2                 | 5.6 $\times 10^8$ | 160±20     |
| MM1                      | 220±50     | 3.9±0.8              | 3.0 $\times 10^5$ | N/A        |
| de blunt                 | 100±30     | 2.5±0.4              | 4.2 $\times 10^5$ | N/A        |

**Fig. 4. Analysis of premelted hTdp1 double strand blunt substrates.** Double strand blunt (ds blunt) substrates containing zero (MM0, identical to double strand blunt substrate described in the legend for Fig. 3), one (MM1), or two (MM2) mismatches at the 3′-end of the phosphotyrosine-containing oligonucleotide were incubated with hTdp1, and reaction velocities were determined as a function of substrate concentration. A, the hybridized substrates were resolved on a non-denaturing polyacrylamide gel before and after incubation with hTdp1. The time of each reaction and the position of double-stranded DNA containing a 3′-phosphotyrosine (ds 3′Y) or 3′-phosphate (ds 3′PO4) are indicated. Single-stranded 3′-phosphotyrosine DNA was also resolved on the gel as a control. B, observed reaction velocities are plotted as a function of substrate concentration. All reactions contained identical enzyme concentrations. $C, k_{cat}$ and $K_M$ values were obtained by fitting the data to the Michaelis-Menten equation.
the same size (16-mer) but lacking a 3'-phosphotyrosine residue (3'-hydroxyl). The results in Fig. 5B show that Tdp1 also binds this molecule with very low affinity. It is important to note that the binding studies must be performed at pH 8 or greater because of the spectral properties of fluorescein; the kinetic experiments were performed at pH 6.5. This prevents a direct comparison of $K_d$ and $K_m$ values (Table I). However, the most important comparison is between the different substrates in each experiment, and taken together, the results show that efficient Tdp1 cleavage of a substrate correlates with the high affinity binding of that substrate.

**DISCUSSION**

The results presented here show that Tdp1 does not have a kinetic or binding preference for single strand DNA over blunt end duplex DNA and are therefore in disagreement with the proposed binding model of Tdp1 and DNA. Multiple crystal structures have been completed of hTdp1 in complex with single-stranded DNA substrate mimics of 3–6 nucleotides in length (26–28). In these structures, the active site nucleophile of hTdp1 lies in the middle of a distinct channel. In one direction, the channel is well suited to protein interactions because it is relatively wide, deep, and negatively charged. In the other direction, the channel is narrow and positively charged, forming the presumed binding site for DNA (27, 28). Although oligonucleotides of up to six bases were used in the crystal structure determinations, only 3 nucleotides (4 phosphodiester) were visualized in the electron density of these structures. This may be due to disorder of the single-stranded DNA in the binding cleft or caused by the occlusion of the binding site by symmetry-related protein molecules in the crystal lattice. The 3'-terminal nucleotides are visible with low B-factors, and 3 conserved residues in Tdp1 (Ser-400, Ser-403, Ser-518) are in position to make hydrogen bonds to the phosphodiester backbone (26).

The observation that blunt double-stranded DNA is efficiently cleaved by Tdp1 and binds to Tdp1 with high affinity prompted us to superimpose the phosphate backbone of double-stranded DNA onto the sugar phosphate backbone of the single-stranded DNA observed in the hTdp1 complex. This superposition allowed the complementary strand to be modeled without clashes or structural modification of the enzyme, although the duplex was modeled as a rigid B-form duplex. In this new model (Fig. 6), only the first three bases sit within the basic cleft proposed to be the DNA-binding pocket. The axial rise of the DNA duplex wraps the continuing helix away from the enzyme surface instead of into the proposed narrow DNA cleft. The blunt end of the duplex contacts the protein surface at conserved residues Ser-400, Pro-461, and Trp-590. Most importantly, the DNA duplex has excellent shape complementarity with the protein, and the side chains of residues Arg-232 and Lys-231 are in close proximity to the opposing DNA strand. One minor contravening observation to this model is a close proximity (4.5 Å) between Glu-233 and a phosphate of the opposing DNA strand. The negatively charged Glu-233 would be expected to repulse a negatively charged phosphate. However, this repulsive force could be overcome either because it is not significant or via changes in the duplex structure, which is modeled with rigid B-form properties. Also, it is possible that
this contact could be mediated via a metal ion, although no dependence on metal ions has been experimentally demonstrated for duplex binding by Tdp1. It is also interesting to note that tryptophan 590 could stack upon the 5′-terminal base of the complementary strand. This stacking interaction would stabilize the 3′-terminal base of the cleaved phosphor-tyrosyl phosphodiester. This would explain why Tdp1 has a profound preference for blunt end double-stranded DNA and single-stranded DNA over nicked DNA.

At what stage during DNA repair does Tdp1 act? The results presented here clearly show a preference for single-stranded and double-stranded blunt DNA substrates over nicked or tailed substrates. These substrates have significantly higher specificity constants (k_cat/K_m); this value is important because it reflects the rate at which a substrate will get cleaved at low concentrations that are expected to occur in vivo. The same trends were observed in the Tdp1 binding assay; Tdp1 binds single-stranded and double-stranded blunt substrates with significantly higher affinity (lower K_m). Because single-stranded DNA is not expected to be a common substrate in vivo, we propose that double-stranded blunt DNA is the natural substrate in vivo. If this is the case, these results clearly suggest that Tdp1 acts after the topoisomerase I-mediated damage is converted to a double strand break (DSB).

The gene encoding yeast TDP1 was originally discovered by screening mutant yeast for sensitivity to the Top1 poison camptothecin. In yeast, abortive topoisomerase I-DNA complexes are converted to DSBs after collision with the replication machinery, and camptothecin poisoning is S-phase-specific. It has also been shown that TDP1 and RAD52 act in the same epistasis group that clearly defines the role of Tdp1 in a DSBR pathway. Although it is important to note that there are other mechanisms to repair abortive topoisomerase I-DSBs such as those reported to arise from collision of RNA polymerase with overlapping topoisomerase I-SSBs (36). It has been proposed that XRCC1 could play a role in the repair of DSBs (35). A protein partner of XRCC1, PARP-1, binds both SSBS and DSBs (37), and in a non-homologous end-joining-defective background, the additional disruption of PARP-1 results in embryonic lethality (38). This has raised the possibility that P ARP-1 fulfills a backup role during DSBR, by binding and recruiting XRCC1 and other SSBS proteins to DSBs that are not repaired by classical non-homologous end-joining (35). This might be important if non-homologous end-joining becomes saturated or is absent or perhaps at a subset of DSBs at which this process is inefficient.

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Substrate Specificity of Tyrosyl-DNA Phosphodiesterase I (Tdp1)
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J. Biol. Chem. 2005, 280:22029-22035.
doi: 10.1074/jbc.M502148200 originally published online April 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M502148200

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