

Design and synthesis of fluorescent substrates for human tyrosyl-DNA phosphodiesterase I

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Received June 30, 2004; Revised and Accepted August 10, 2004

ABSTRACT

Tyrosyl-DNA phosphodiesterase 1 (Tdp1) is a DNA repair enzyme that acts upon protein–DNA covalent complexes. Tdp1 hydrolyzes 3'-phosphotyrosyl bonds to generate 3'-phosphate DNA and free tyrosine *in vitro*. Mutations in Tdp1 have been linked to patients with spinocerebellar ataxia, and over-expression of Tdp1 results in resistance to known anti-cancer compounds. Tdp1 has been shown to be involved in double-strand break repair in yeast, and Tdp1 has also been implicated in single-strand break repair in mammalian cells. Despite the biological importance of this enzyme and the possibility that Tdp1 may be a molecular target for new anti-cancer drugs, there are very few assays available for screening inhibitor libraries or for characterizing Tdp1 function, especially under pre-steady-state conditions. Here, we report the design and synthesis of a fluorescence-based assay using oligonucleotide and nucleotide substrates containing 3'-(4-methylumbelliferone)-phosphate. These substrates are efficiently cleaved by Tdp1, generating the fluorescent 4-methylumbelliferone reporter molecule. The kinetic characteristics determined for Tdp1 using this assay are in agreement with the previously published values, and this fluorescence-based assay is validated using the standard gel-based methods. This sensitive assay is ideal for kinetic analysis of Tdp1 function and for high-throughput screening of Tdp1 inhibitory molecules.

INTRODUCTION

DNA topoisomerases are ubiquitous enzymes that catalyze changes in DNA topology by altering the linkage of DNA strands (1). Topoisomerase I (TopI) uses an active site tyrosine residue to cleave one strand of DNA forming a 3'-phosphotyrosine intermediate. TopI is the only eukaryotic enzyme to form such a 3'-phosphotyrosyl covalent bond. 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 be accumulated in the presence of naturally occurring DNA damage, such as nicks (3), abasic sites (4), modified bases (5), modified sugars (6) or as a result of exposure to a variety of chemotherapeutic drugs such as camptothecin (7,8).

The *Tdp1* gene was originally isolated in a camptothecin sensitivity screen of *Saccharomyces cerevisiae* (9). Camptothecin is a well-characterized chemotherapeutic agent that stabilizes the transient TopI–DNA 3'-phosphotyrosyl intermediate. There are two camptothecin derivatives that are currently used clinically to treat human cancer (7,8). These TopI 'poisons' presumably overwhelm the ability of endogenous repair mechanisms to reverse these cytotoxic lesions and therefore result in the death of these rapidly dividing cells. Over-expression of tyrosyl-DNA phosphodiesterase 1 (Tdp1) in human cells conveys resistance to high levels of camptothecin and it is expected that the inhibition of Tdp1 activity will convey sensitivity to camptothecin (10). Tdp1 inhibitors may therefore potentiate the activity of clinically approved TopI poisons, or may be effective anti-cancer drugs due to the natural accumulation of covalent TopI–DNA complexes. It is important to emphasize however that *Tdp1* does not appear to be the only mechanism for the repair of TopI covalent complexes in yeast, and the same may prove to be true for human cells (11,12). The camptothecin sensitivity screen that originally identified the yeast *TDP1* gene required a *rad9* deficient background (9). The *RAD9* gene is essential for function of the DNA damage checkpoint (13). Indeed, all yeast genetic screens of *tdp1* require at least one additional mutation in order to detect the loss of *tdp1*. Using a multiple mutation experiment, Vance *et al.* (12) showed that the site-specific endonucleases *RAD1*–*RAD10* also repair TopI adducts, although this activity is highly dependent on the substrate structure.

Tdp1 represents the prototype of enzymes that act on protein–DNA complexes (14), and understanding the structure and function of Tdp1 has received a growing amount of

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attention. Several crystal structures of human Tdp1 in the presence and absence of substrate mimics have been published previously (15–18) and the roles of several catalytic residues have recently been identified (19). Functional studies have been limited by the difficulty in assaying Tdp1 activity, especially under pre-steady-state conditions. Tdp1 activity has traditionally been measured by synthesizing 3'-phosphotyrosine or 3'-phosphotyrosine analog [such as 3'-(4-nitro)phenyl phosphate] oligonucleotides or by isolating small peptide fragments covalently linked to DNA, and resolving the reaction product (3'-phosphate DNA) from substrate on a polyacrylamide gel (19–21). 3'-(4-Nitro)phenyl phosphate DNA is a chromagenic substrate that has been used previously; however, spectrophotometric detection of 4-nitrophenol requires very high concentrations because of the relatively poor extinction coefficient (22). As a result, 3'-(4-nitrophenyl) phosphate DNA can only be used to assay Tdp1 at concentrations much higher than the apparent K_M and is therefore not generally useful for most kinetic studies. Here, we show that 7-hydroxy-4-methyl-coumarin (also known as 4-methylumbelliferone) can act as a tyrosine mimic for Tdp1, and oligonucleotides containing a 3'-phospho-(4-methylumbelliferone) group are efficient substrates for human Tdp1 *in vitro*. These derivatized DNA molecules [e.g. 3'-(4-methylumbelliferone)-phosphate DNA (DNA-MUP)] are not fluorescent; however, Tdp1-mediated cleavage generates 3'-phosphate DNA and fluorescent-free 4-methylumbelliferone. Analysis of the DNA-MUP substrate in both the fluorescent and gel-based assay demonstrates that both assays yield very similar K_M and k_{cat} values. In addition, these values are very similar to those obtained with oligonucleotides containing a single 3'-phosphotyrosine residue (19). Taken together, these results demonstrate that the fluorescent assay is an accurate measure of Tdp1 activity and is useful for determining Tdp1 function *in vitro*.

We have also synthesized a single thymidine nucleotide containing a 3'-phospho-(4-methylumbelliferone) moiety. This mononucleotide substrate can be readily synthesized in gram quantities, and is also cleaved by Tdp1 to generate the same fluorescent reporter. It has been previously shown that Tdp1 has a much higher affinity for oligonucleotide substrates than mononucleotide substrates (23), and our analysis of the fluorescent substrates results in the same conclusion. This is expected since crystal structures have shown that three conserved Tdp1 residues make specific hydrogen bond contacts with substrate bridging phosphodiester groups (17). Together, these substrates provide extremely useful tools for measuring Tdp1 activity, especially under pre-steady-state conditions, and for the high-throughput screening of new Tdp1 inhibitors.

MATERIALS AND METHODS

Synthesis of oligonucleotide substrate (DNA-MUP)

The synthesis of DNA-MUP is shown as a schematic diagram in Figure 1. A 3'-phosphate oligonucleotide d(GAA-TAACTTCGTATAAp) was synthesized on an ABI 392 DNA synthesizer using a 3'-phosphate resin (Glen Research). The 3'-phosphate oligonucleotide was purified as described previously (22). The post-synthetic approach for derivatizing the 3' end of oligonucleotides was adapted from (24). Briefly, the

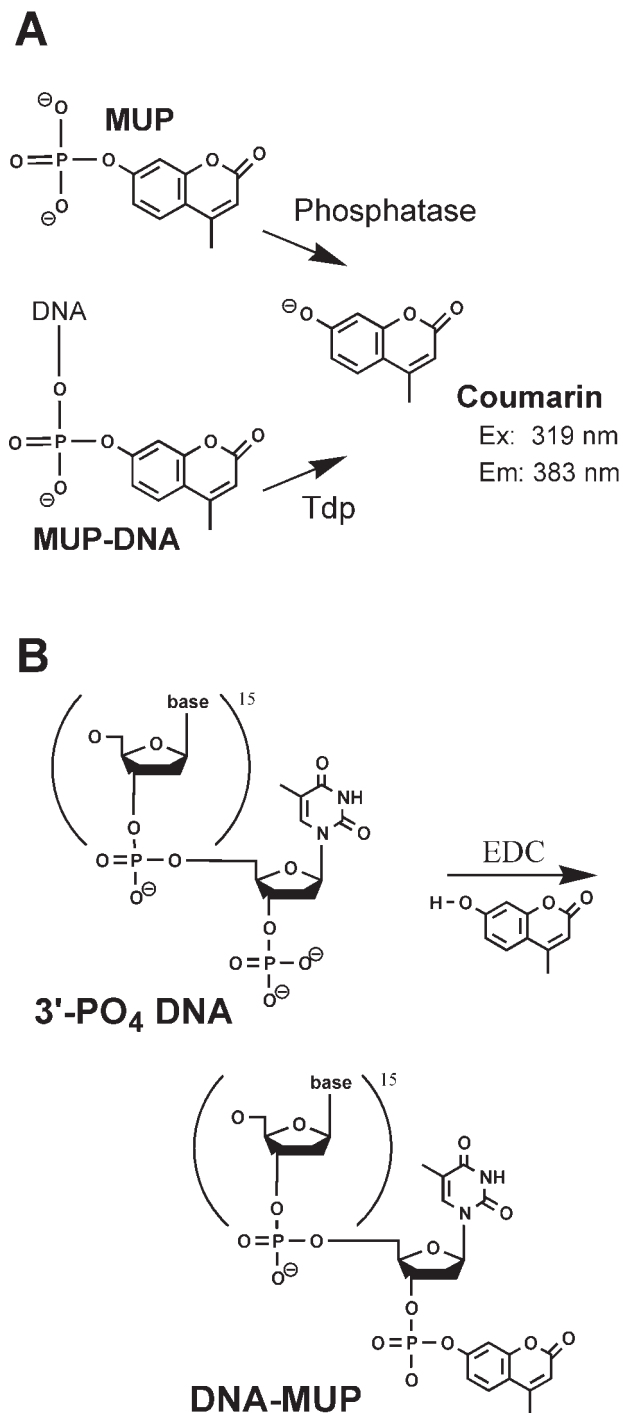


Figure 1. (A) Rationale for DNA-MUP substrate for Tdp1 studies. MUP is a well-characterized substrate for tyrosine phosphatase assays. Tyrosine phosphatases cleave MUP to liberate fluorescent free coumarin, allowing fluorometric quantitation of enzyme activity. We reasoned that the analogous phosphodiester substrate (DNA-MUP) could be used to assay tyrosine phosphodiesterases, specifically Tdp1. (B) DNA-MUP synthesis. Coumarin is condensed onto 3'-phosphate DNA in one step using EDC to generate DNA-MUP.

oligonucleotide was dissolved in 150 μ l of 100 μ M MES (pH 5.5) and 2 mM $MgCl_2$. Next, 0.048 g of solid 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC; Sigma) and 150 μ l of 2 M 4-methylumbelliferone (Aldrich)

in dimethyl sulfoxide (DMSO) were added sequentially. The reaction was shaken vigorously in the dark at room temperature for 12 h. Excess fluorophore was removed by two ethyl acetate extractions, and the DNA was isolated from the aqueous layer by ethanol precipitation. The resulting pellet was resuspended in 100 μ l of water and the derivatized DNA-MUP was purified by anion exchange chromatography on a Hitachi analytical high-performance liquid chromatography (HPLC) using a DNAPac PA-100 4 mm \times 250 mm column (Dionex, Sunnyvale, CA) at 1 ml/min, 5–60% of 2 M sodium chloride gradient (buffered with 20 mM sodium phosphate, pH 7.0) over 15 min. Peak fractions were pooled, concentrated by ethanol precipitation and stored at -20°C in 5 mM MES (pH 6.0). Product identity was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS) (data not shown).

Synthesis of mononucleotide substrate (dT-MUP)

The synthesis of dT-MUP is shown as a schematic diagram in Figure 4. An aliquot of 5 g MUP was resuspended in 40 ml of anhydrous pyridine (Aldrich) under argon, and 1 M equivalent of POCl_3 (Aldrich) was added under argon. After 1 h at room temperature, 1 M equivalent of 5'-(dimethoxytrityl)-thymidine mononucleoside (Chemgenes) and 3 M equivalents of *N,N*-diisopropylethylamine (Aldrich) were added simultaneously. After stirring at room temperature for 18 h, the reaction was quenched with the addition of 1 ml of water. The reaction was dried *in vacuo* and the products were purified by silica gel chromatography (5–15% methanol in dichloromethane). Pure fractions were pooled, dried and then resuspended in 10 ml of 80% glacial acetic acid/ethanol (1:1) to remove the 5'-DMT group. The nucleotide was precipitated by adding barium acetate to 20% (w/v) and isolated by centrifugation. The precipitate was washed with 20 ml of ethanol and resuspended in 2 ml of water. The triethylammonium salt was formed by adding an excess of Dowex-50 previously saturated with triethylammonium acetate. The final solution was filtered, and 50% sodium hydroxide (w/v) was added dropwise to bring the final pH to 6.0. Purified dT-MUP was stored at -20°C . Product identity was confirmed by MALDI-TOF MS (data not shown).

Preparation of human Tdp1

Human Tdp1 was purified as described previously (19). In all experiments, a variant of human Tdp1 containing an N-terminal deletion was used. This variant has been used to obtain several different crystal structures (15–17,19) and is fully functional (19,21).

Fluorescence-based enzyme assays

The wild-type Tdp1 was concentrated to 69 μM for use in the mononucleotide substrate experiments, or diluted to 500 pM for use in the oligonucleotide substrate experiments. Enzyme activity assays were performed in 50 mM Tris-HCl (pH 8.0), 80 mM KCl, 2 mM EDTA, 1 mM dithiothreitol (DTT), 40 $\mu\text{g/ml}$ BSA and 10% DMSO. The reactions were monitored in white, flat bottom, non-treated, 96-well polystyrene assay plates (Costar). Measurements were taken in a POLARstar plate reader (BMG) at 37°C using a 355 nm excitation filter and a 460 nm emission filter. The absolute concentration of

product was extrapolated from a standard curve of fluorescence versus 4-methylumbelliferone.

Gel-based enzyme assays

Wild-type Tdp1 activity assays were performed in 50 mM Tris-HCl (pH 7.8), 80 mM KCl, 2 mM EDTA, 1 mM DTT, 40 $\mu\text{g/ml}$ BSA and 10% DMSO at 37°C . Enzyme stocks were diluted in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM DTT, 10% glycerol and 500 $\mu\text{g/ml}$ BSA. Activity assays contained a final Tdp1 concentration of 3.4 pM. DNA-MUP cleavage substrates were 5'-radiolabeled with [α - ^{32}P]ATP (PerkinElmer) using T4 polynucleotide kinase (New England Biolabs) at 37°C for 15 min, followed by 10 min at 90°C to denature the kinase. All reactions were performed at 37°C in 96-well v-bottom reaction plates and quenched by the addition of an equal volume of 8 M urea, 0.05% SDS, 30% glycerol, 0.25% bromophenol blue and resolved on 20% polyacrylamide sequencing gels. The concentration of 3'-phosphate DNA product was determined by measuring the fraction of substrate converted into 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 <20% of initial substrate concentrations were used, all lines extrapolated to zero product formed 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 and treated independently. Apparent K_M and V_{max} values were determined by fitting the initial velocity versus substrate concentration to the Michaelis equation, $v = (V_{\text{max}} * [\text{S}]) / (K_M + [\text{S}])$. Very similar values were obtained from Eadie-Hofstee plots of the same data (data not shown).

RESULTS

Previous studies have shown that the yeast and human Tdp1 can recognize and cleave a variety of functional groups linked to the 3' end of DNA. For example, a small fragment of human Top1 or a single tyrosine residue are efficient substrates for human Tdp1 (20,21). In addition, yeast Tdp1 can cleave a single tyrosine residue or several amino acids linked to DNA with nearly identical k_{cat}/K_M values (25). Finally, we have recently shown that oligonucleotides containing a (4-nitro-phenyl)-phosphate or (4-methyl-phenyl)-phosphate group are efficient substrates for human Tdp1 *in vitro* (19). Because of the diversity of moieties that can be cleaved by Tdp1, we reasoned that DNA-MUP could also be cleaved by Tdp1. MUP is a well-characterized substrate for assaying tyrosine phosphatases (26–28). Hydrolysis of the phosphate group generates fluorescent 4-methylumbelliferone (coumarin) and we reasoned that the analogous substrate could be used to assay tyrosine phosphodiesterases (see Figure 1A).

DNA-MUP was synthesized (Figure 1B) by derivatizing 3'-phosphate oligonucleotides with 4-methylumbelliferone (coumarin) using the water soluble condensing agent 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC). By optimizing the pH (5.5) and the concentration of coumarin (0.5 M), we were able to convert $\sim 75\%$ of single-strand oligonucleotides into DNA-MUP in 12 h. An oligonucleotide containing a 3'-terminal thymidine residue is shown in Figure 1B, but

we have also derivatized oligonucleotides containing 3'-terminal adenosine, cytosine and guanine nucleotides with similar efficiencies (data not shown). The DNA-MUP product was purified away from 3'-phosphate DNA by anion exchange or reverse phase HPLC, and the identity of the final product was confirmed by MALDI-TOF MS (data not shown).

To determine if DNA-MUP is a substrate for human Tdp1, we first used a traditional polyacrylamide gel-based assay. As described above, Tdp1 activity has been monitored using 5' end labeled oligonucleotides containing a 3'-tyrosine or tyrosine analog; the hydrolysis reaction liberates 3'-phosphate DNA which migrates slightly faster in a polyacrylamide gel due to an increase in charge and decrease in molecular weight (20). The results in Figure 2A show that Tdp1 converts

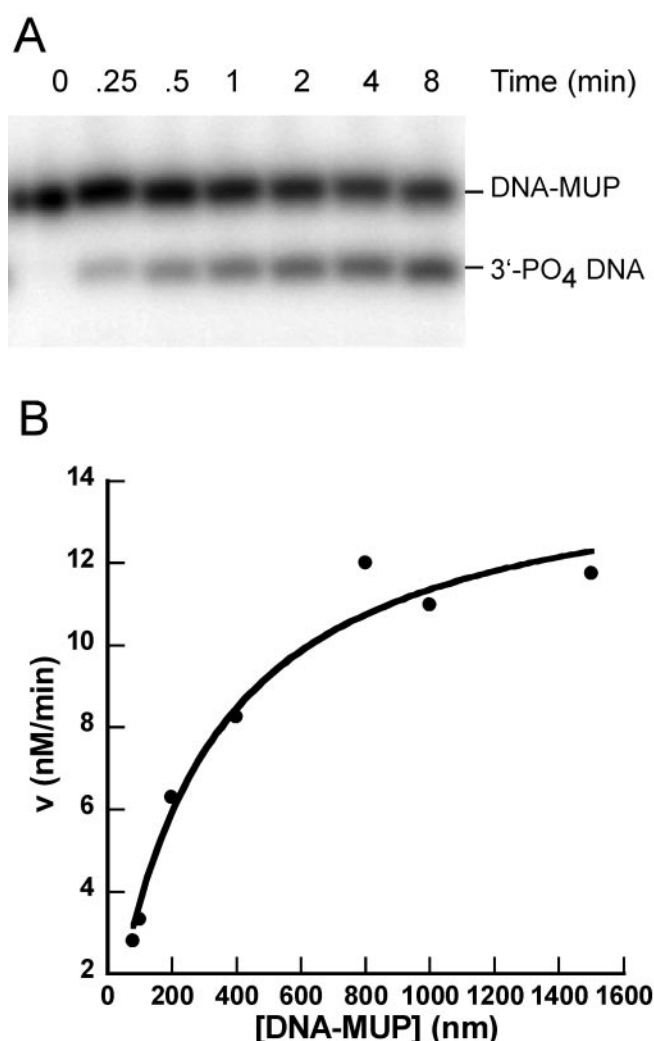
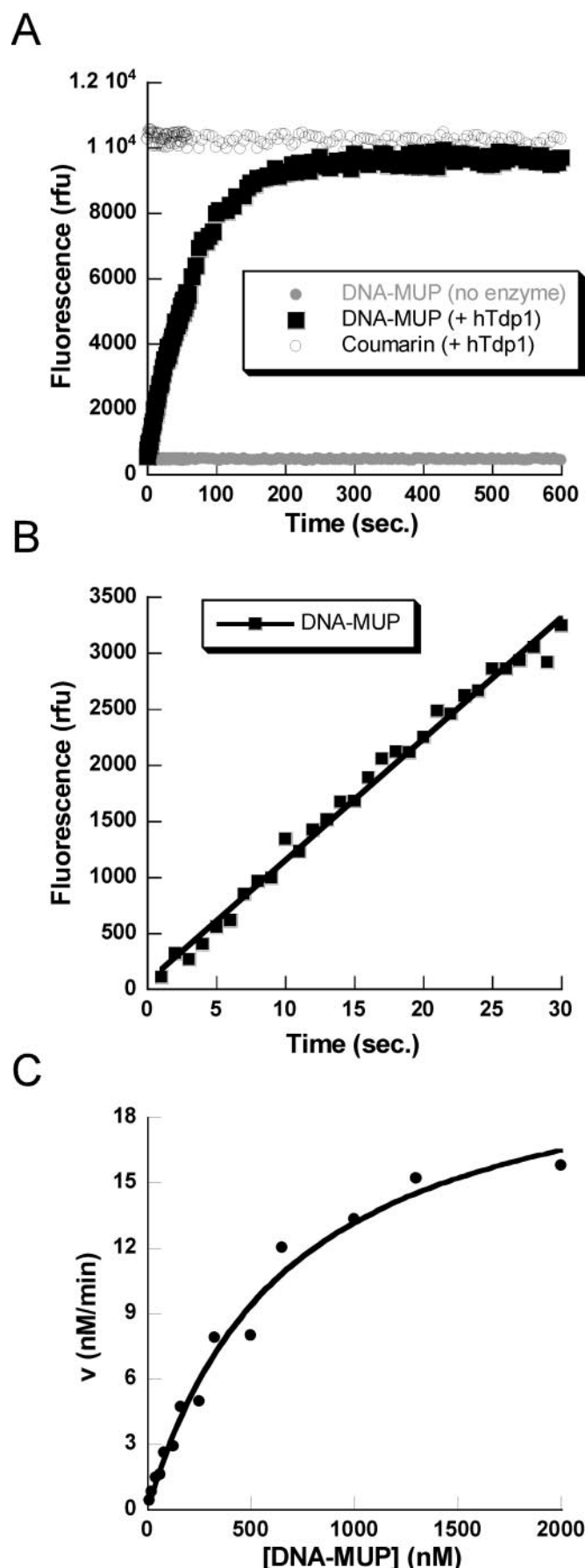


Figure 2. (A) Gel-based Tdp1 activity assay. 5'-Radiolabeled DNA-MUP was reacted with 3.4 pM Tdp1 at 37°C and timepoints were quenched at the indicated reaction time. The reaction products were resolved on a 20% acrylamide gel containing 8 M urea, and the resulting autoradiogram is shown. The positions of DNA-MUP substrate and 3'-phosphate reaction product are indicated on the right. (B) Kinetic analysis of gel-based Tdp1 activity assay. Reaction velocities were determined as described in Materials and Methods, and are graphed as a function of substrate concentration. The data were fitted to the Michaelis equation (see Materials and Methods), and the resulting V_{\max} and K_M values are shown in Table 1.

DNA-MUP into a faster migrating species as a function of time. This reaction product co-migrates precisely with control 3'-phosphate oligonucleotide (data not shown). To determine how well Tdp1 is recognizing the DNA-MUP substrate, reaction velocities were measured as a function of DNA-MUP concentration. These results (Figure 2B) show that DNA-MUP can saturate Tdp1 with an apparent K_M value of 300 ± 60 nM. This value is very similar to K_M values obtained with oligonucleotides containing a 3'-tyrosine residue (500 nM) under similar conditions (19). The observed V_{\max} value was 15 ± 1 nM min⁻¹.

If Tdp1 is generating 3'-phosphate DNA in these experiments, then coumarin should also be generated as a result of the hydrolysis reaction. We therefore repeated the experiments using unlabeled DNA-MUP and measured the fluorescence as a function of time. The results in Figure 3A show that when DNA-MUP is incubated with Tdp1, a time-dependent fluorescent signal is observed. The maximum amount of fluorescence presumably occurs when Tdp1 converts all of the DNA-MUP into 3'-phosphate DNA and coumarin; and as expected the amount of fluorescence is roughly equal to the amount of fluorescence observed when coumarin is added at the same concentration instead of DNA-MUP. The slight decrease in fluorescence at later time presumably results from bleaching of the fluor; however, at early times the degree of bleaching is negligible and the initial rate of fluorescence should accurately reflect the rate of product formation. If DNA-MUP is incubated in the absence of Tdp1, a small fluorescent signal that does not change with time is observed and represents the background fluorescence of the DNA-MUP reaction. The results in Figure 3B show that when this background is subtracted from the enzyme-dependent fluorescence, the signal extrapolates to zero at the beginning of the reaction.

Because we predict that this fluorescence assay and the gel-based assay are monitoring the same reaction, we expect both assays should yield very similar K_M and k_{cat} values. In order to determine the amount of product formed as a function of time in the fluorescence assay, we first generated a standard curve of fluorescence versus coumarin concentration (data not shown). This standard curve was then used to determine the concentration of coumarin generated as a function of time (velocity, nM min⁻¹) from the fluorescence plots (Figure 3A). Initial reaction velocities were subsequently measured at different DNA-MUP concentrations and the resulting velocity versus substrate concentration curve is shown in Figure 3C. As expected, DNA-MUP saturates the enzyme with apparent K_M value of 700 ± 100 nM, and an observed V_{\max} value of 22 ± 2 nM min⁻¹. A comparison of k_{cat} and K_M values (Table 1) shows that both assays give very similar values. The small differences in kinetic constants obtained from the two assays may result from differences in quantitation (PhosphorImager analysis versus fluorescence), systematic differences in instrumentation (direct determination versus quenching reactions by pipetting) or small differences in temperature (automated temperature controlled plate reader versus manually operated heat block). Because of the many potential differences in the two assays, we conclude that the observed kinetic constants are very similar and that both assays are measuring the same enzymatic reaction. Taken together, these results demonstrate that DNA-MUP is a valid substrate for assaying Tdp1 function *in vitro*.



Previous studies have shown that single nucleotides can be substrates for Tdp1, although Tdp1 has a much higher affinity for oligonucleotide substrates (20,23). We proposed that a single thymidine nucleotide linked to 4-methylumbelliferone through a 3'-phosphodiester (dT-MUP) would be a substrate for human Tdp1. Nucleotides can be obtained in much higher quantities than oligonucleotides, and the protocol for synthesizing dT-MUP (outlined in Figure 4) does not require a post-synthetic derivatization step. Coumarin is first activated with phosphorous oxychloride (step 1) and then conjugated to the 3'-hydroxyl of 5'-protected thymidine (step 2). The phosphodiester is generated by the addition of water, and the 5'-dimethoxytrityl protecting group is removed by acid treatment (step 3). Using this method, we obtained 2 g of dT-MUP (35% overall yield).

To determine if dT-MUP is a substrate for Tdp1, we measured fluorescence as a function of enzyme concentration. In the absence of Tdp1, no detectable increase in fluorescence was observed (data not shown). However, increasing concentrations of Tdp1 did result in an increase in the rate of fluorescence (Figure 5A). An analysis of the initial rates of hydrolysis does show a clear dependence upon Tdp1 concentration (Figure 5B).

In our attempts to determine K_M and k_{cat} values of dT-MUP using human Tdp1, we were unable to saturate the enzyme. Experiments were performed at concentrations as high as 100 μ M dT-MUP, and at these concentrations the substrate begins to quench fluorescence of coumarin (data not shown). This result may be expected since X-ray crystal structure models of human Tdp1 bound to an oligonucleotide (17) show that the enzyme makes several contacts with nucleotides upstream of the 3'-terminal nucleotide and human Tdp1 would therefore be expected to bind nucleotides with greatly reduced affinity. It is likely that the K_M for dT-MUP by Tdp1 is in the millimolar range and therefore cannot be measured accurately in this assay.

This fluorescence-based assay may be very useful for identifying Tdp1 inhibitors. To test this possibility, Tdp1 activity was assayed in the presence of vanadate, a well-characterized non-specific inhibitor of phosphoryl transfer reactions; vanadate is known to inhibit Tdp1 and has been useful in Tdp1 co-crystallization studies (17,18). Cleavage of DNA-MUP was assayed in the absence and presence of 1 or 50 mM sodium orthovanadate. Fluorescence was monitored as described previously and the results are graphed in Figure 6. The presence of 50 mM

Figure 3. (A) DNA-MUP is cleaved by Tdp1. DNA-MUP was reacted with Tdp1 at 37°C and time resolved fluorescence was measured through a 355 nm excitation filter and a 460 nm emission filter. DNA-MUP (500 nM; filled squares) or coumarin (500 nM; open circles) were incubated in the presence of Tdp1 (3.4 pM) and the fluorescent signal is plotted as a function of time. Incubation of DNA-MUP without the addition of Tdp1 is also plotted (filled circles). (B) Fluorescence-based Tdp1 activity assay. The fluorescent signal due to Tdp1 hydrolysis was obtained by subtracting the fluorescent signal in the absence of Tdp1 from the fluorescent signal observed in the presence of Tdp1. This value is plotted as a function of time. Only the first 30 s of the time course is shown. (C) Kinetic analysis of fluorescence-based Tdp1 activity assay. A range of DNA-MUP substrate concentrations were reacted with 3.4 pM Tdp1 and resulting Tdp1-dependent fluorescence was measured. The relative fluorescence units (r.f.u.) were converted into concentration of product formed over time, yielding true reaction velocities (nM min^{-1}). Reaction velocities are graphed as a function of substrate concentration and fitted to the Michaelis equation. The resulting V_{max} and K_M values are shown in Table 1.

Table 1. Summary of Tdp1 kinetic analysis by gel-based and fluorescence-based methods

Readout	k_{cat} (min^{-1})	K_M (nM)	k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$)
Fluorescence	6500 ± 400	700 ± 100	1.5×10^8
Gel-based	4300 ± 300	300 ± 60	2.4×10^8

Kinetic values were extracted from the graphs shown in Figures 2B (gel-based) and 3C (fluorescence-based). Human Tdp1 displays nanomolar affinity for the DNA-MUP substrate according to both assays, and the resultant K_M (nM) and V_{max} (nM min^{-1}) are within a 2-fold of each between the two assays. k_{cat} values (min^{-1}) were determined by dividing the V_{max} using the enzyme concentration (3.4 pM). Specificity constants (k_{cat}/K_M) are expressed as $\text{M}^{-1} \text{s}^{-1}$.

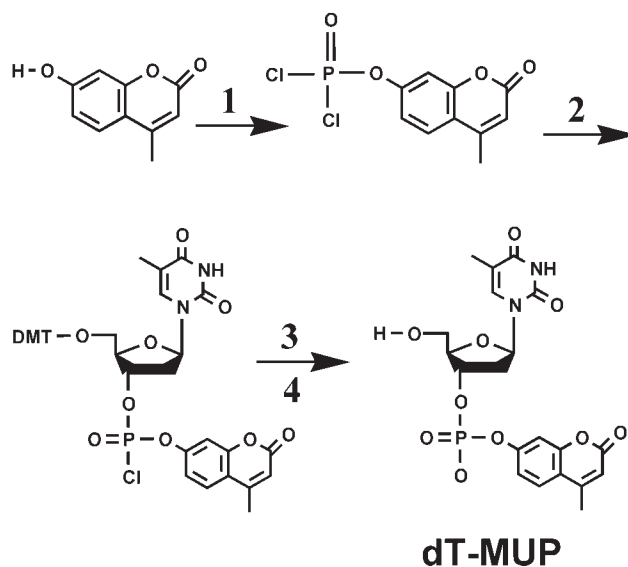


Figure 4. Reaction scheme for the synthesis of mononucleotide substrate. Details of the synthesis are described in Materials and Methods. 1, 4-methylumbelliferone (coumarin) and POCl_3 in anhydrous pyridine; 2, excess of N,N -diisopropylethylamine and 5'-(dimethoxy-trityl)-thymidine mononucleoside; 3, aqueous 80% glacial acetic acid; and 4, barium acetate precipitation.

vanadate completely eliminates Tdp1 activity, and 1 mM vanadate partially inhibits Tdp1 activity.

DISCUSSION

Tdp1 represents a unique DNA repair enzyme that cleaves cytotoxic protein–DNA covalent complexes. Tdp1 hydrolyzes 3'-tyrosine phosphodiesterases to generate free tyrosine and 3'-phosphate DNA. Based on fluorescent substrates known to be cleaved by tyrosine phosphatases, we designed fluorescent substrates that could be cleaved by Tdp1, a tyrosine phosphodiesterase. Here, we describe the synthesis and utility of oligonucleotide and nucleotide fluorescent substrates for assaying Tdp1 activity *in vitro*. The oligonucleotide substrate (DNA-MUP) is cleaved very efficiently by Tdp1 with an observed specificity constant of $\sim 1 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$. This apparent second-order rate constant is near the rate of diffusion and is essentially identical to values obtained with 3'-tyrosine or 3'-tyrosine analog substrates (19). This result is important because it has been argued that Tdp1 specifically recognizes

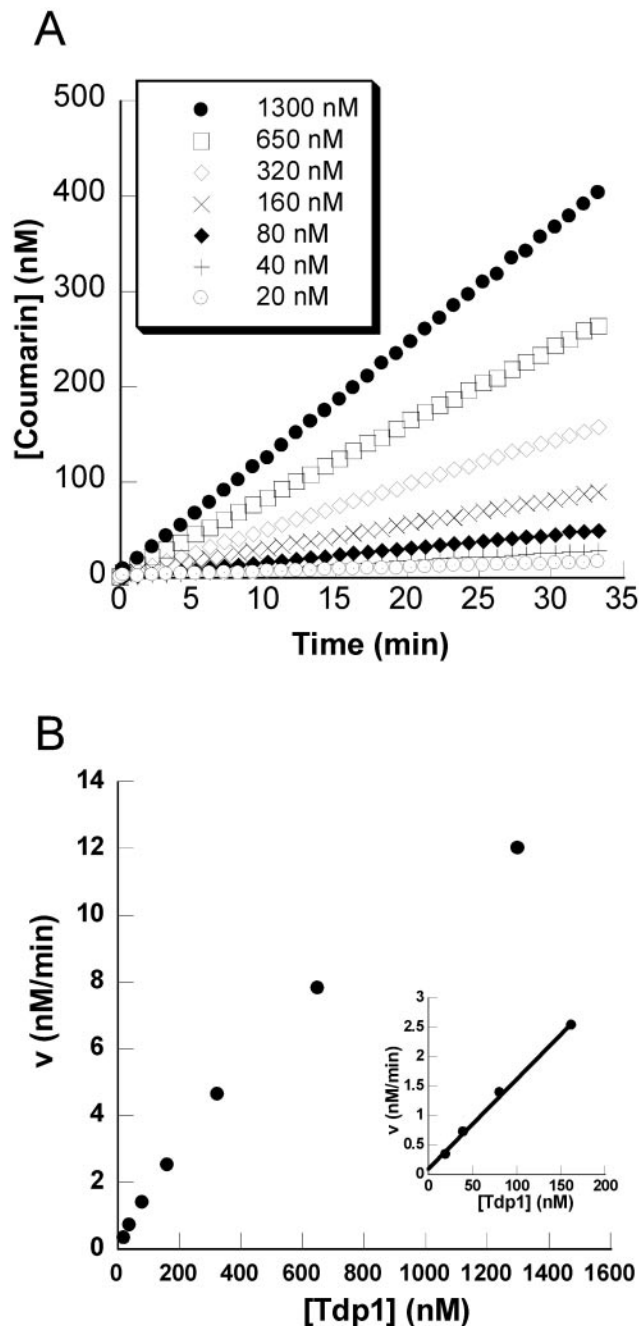


Figure 5. (A) Human Tdp1 concentration-dependent reaction rates. dT-MUP mononucleotide substrate ($1 \text{ }\mu\text{M}$) was reacted with increasing concentrations of Tdp1 at 37°C and resulting fluorescence was converted into concentration of coumarin product formed over time. (B) Analysis of Tdp1 concentration-dependent reaction rates. Reaction velocities obtained from linear regression analysis of data presented in (A) are graphed as a function of enzyme concentration. Under multiple turnover conditions, one would expect a linear relationship between the observed velocity and enzyme concentration. The results show that when the enzyme concentration is much lower (20, 40, 80 and 160 nM) than the substrate concentration ($1 \text{ }\mu\text{M}$), the rate of cleavage is proportional to enzyme concentration (inset). In addition, the rate of cleavage extrapolates to zero in the absence of Tdp1.

a proteolyzed fragment of TopI linked to DNA (16,17). A variety of genetic studies have demonstrated the importance of Tdp1 for the repair of TopI-induced double-strand breaks (9,11,12,29). However, the fact that Tdp1 can efficiently

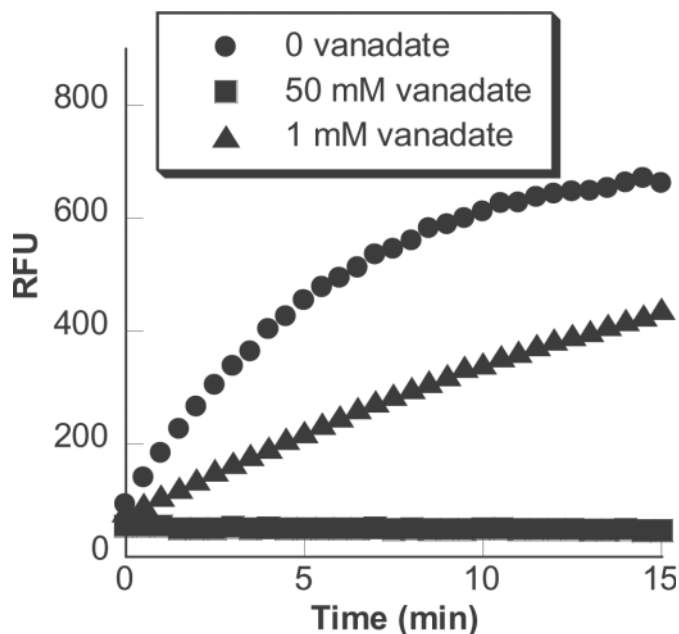


Figure 6. Inhibition of Tdp1. Tdp1 (50 pM) was reacted with DNA-MUP (100 nM) in the absence and presence of 1 or 50 mM sodium orthovanadate. Relative fluorescent units (r.f.u.) are plotted as a function of time (min) without background subtraction.

cleave a wide variety of moieties, some of which Tdp1 would not be expected to encounter *in vivo* (coumarin), suggests that Tdp1 function does not require specific contacts with TopI. Instead, Tdp1 may only recognize the DNA and the 3'-phosphodiester of the TopI-DNA covalent complex. It is also possible that the diversity of substrates that can be cleaved by Tdp1 *in vitro*, reflects a diversity of substrates that could be cleaved by Tdp1 *in vivo*. Recent genetic studies have demonstrated that Tdp1 is important for mutation avoidance under normal growth conditions (30), and Tdp1 has been reported to cleave 3'-phosphoglycolate linkages (31) *in vitro*. Clearly, genetic studies are required to determine the additional role(s) of Tdp1 *in vivo*.

The gene encoding Tdp1 was originally identified as a camptothecin hypersensitive mutant in yeast (9). The camptothecins are a class of compounds that bind and stabilize the transient covalent complex formed between TopI and DNA, and two derivatives are currently approved to treat human cancer. This suggests that the inhibitors of Tdp1 could potentiate the activity of these clinically approved drugs or act as anti-cancer drugs by themselves. Such inhibitors could be obtained by screening compound libraries and identifying molecules that inhibit Tdp1 activity *in vitro*. Here, we describe the synthesis of mononucleotide substrates that are ideally suited for this purpose. The synthesis is inexpensive and the substrate can be obtained in gram quantities. Although Tdp1 has a low affinity for dT-MUP, the assay is extremely sensitive and assays can be performed under conditions where enzyme and substrate concentrations are well below K_M . Enzyme concentration can be varied to allow the reaction to be monitored over a few seconds or minutes. Molecules identified in this screen could be characterized using the more biologically relevant oligonucleotide substrate (DNA-MUP).

ACKNOWLEDGEMENTS

We thank Lance Stewart and Mark Gurney for critical reading of the manuscript. A.C.R. was supported by Predoctoral Fellowship 1F31 GM66372-01 from the National Institutes of Health. This research was funded by the Cooperative Planning of the SDSU/UCSD Cancer Partnership (grant CCA92079-01A2) to A.C.R. and grant GM58596 to A.B.B.

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