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Design of a New Fluorescent Oligonucleotide-Based Assay for a Highly Specific Real-Time Detection of Apurinic/Apyrimidinic Site Cleavage by Tyrosyl-DNA Phosphodiesterase 1

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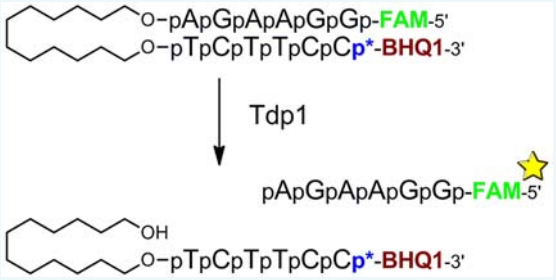
ABSTRACT: Tyrosyl-DNA phosphodiesterase 1 (Tdp1) promotes catalytic scission of a phosphodiester bond between the 3′-end of DNA and the hydroxyl group of a tyrosine residue, as well as cleaving off a variety of other 3′-terminal phosphate-linked DNA substituents. We have shown recently that Tdp1 can initiate an apurinic/apyrimidinic (AP) site repair pathway that is independent from the one mediated by AP endonuclease 1 (APE1). Until recently, there was no method available of tracking the AP-site cleaving activity of Tdp1 by real-time fluorescence assay. In the present study we demonstrate a highly specific real-time detection of the AP-site cleaving activity of Tdp1 which allows one to distinguish it from the activity of APE1 by using a short hairpin oligonucleotide with a 1,12-dodecanediol loop, a 5′-fluorophore, and a 3′-quencher. Specific phosphodiesterase activity of Tdp1, which is usually able to remove quencher from the 3′-end of DNA, was suppressed in our approach by introducing a noncleavable phosphate group mimic between the 3′-end and the quencher. As a nondigestible 3′-phosphate analogue, we have used a new uncharged tetramethyl phosphoryl guanidine (Tmg) group, which is resistant to 3′-phosphodiesterase cleavage.



INTRODUCTION

Tyrosyl-DNA phosphodiesterase 1 (Tdp1), a member of the phospholipase D superfamily, has been identified as the enzyme responsible in cells for specific cleavage of the tyrosyl-DNA phosphodiester linkage formed via a covalent bonding of the phenolic hydroxyl group of the catalytic tyrosine residue of topoisomerase 1 (Top1) and the 3′-terminal phosphate of DNA.[1,2] Due to its central role in this critical DNA repair pathway, Tdp1 has attracted considerable interest as a potential target for anticancer pharmaceutical intervention. A mutation in, or genetic inactivation of, Tdp1 can hypersensitize cells to camptothecin,[3,4] whereas overexpression of the active Tdp1 protein has been shown to result in a significant reduction of camptothecin-induced DNA damage.[5] Tdp1 activity is not limited to the removal of cellular Top1 adducts: the enzyme can also process other 3′-blocking DNA lesions such as 3′-abasic sites or bulky substituents, and use 3′-phosphoglycolate as a substrate.[6−8] Recently, we have shown that Tdp1 can initiate the repair of apurinic/apyrimidinic (AP, abasic) sites independently from the AP endonuclease 1 (APE1) pathway.[9−11] However, among oligonucleotide-based detection methods for enzymatic activities of some DNA repair enzymes that were developed,[12−14] until recently there was no method of tracking the AP-site cleavage activity of Tdp1 by fluorescence assay in real time. The methods that have been adopted so far for monitoring the phosphodiesterase activity of Tdp1 were generally gel-based and tedious.[7,15] An elegant study has recently reported the use of a novel fluorophore-quencher assay to monitor the 3′-phosphodiesterase activity of Tdp1 in cell material.[16] In parallel, a fluorescence assay has been developed for monitoring AP-site cleavage by Tdp1.[17] Finally, Sarah Walker and coauthors have published an oligonucleotide-based fluorescence assay to monitor the 3′-phosphodiesterase activity of Tdp1 that is robust, sensitive, and suitable for high-throughput screening of both fragment and small molecule libraries for potential Tdp1 inhibitors.[18]

Thus, real-time detection of Tdp1 activities may present the easiest and most reliable way of obtaining quantitative analyses in biological samples. However, it is essential that the assay in question allows one to quantify a specific Tdp1-mediated activity such as the Tdp1-mediated AP-site cleavage independently from other potential Tdp1 phosphodiesterase activities and the APE1-mediated AP-site cleavage. In the present study we demonstrate a highly specific real-time detection of the AP-site cleaving activity of Tdp1 that can be reliably separated from the activity of APE1 using a short hairpin oligonucleotide with a 1,12-dodecanediol (Dod) loop, a 5′-fluorophore, and a 3′-

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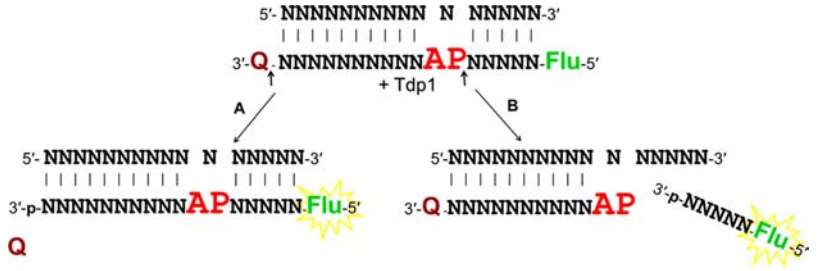

Figure 1. Two possible ways for fluorescent signal appearance during Tdp1-mediated cleavage of an internally quenched oligonucleotide substrate. (A) AP-site cleaving activity of Tdp1. (B) 3′-Phosphodiesterase activity of Tdp1. Cleavage points are indicated by arrows. Flu − fluorophore, Q − dark quencher, AP − apurinic/apyrimidinic site.

quencher. The other potential phosphodiesterase activity of Tdp1 that can remove the quencher from the 3′-end of DNA was specifically suppressed in this approach by introducing a noncleavable phosphodiester group analogue tetramethyl phosphoryl guanidine (Tmg) group between the quencher and the 3′-end of the oligonucleotide.

RESULTS AND DISCUSSION

A report was published recently on identification of inhibitors of endonuclease APE1 using a fluorophore-quencher assay.[19] The substrate was a 17 nt double-stranded (ds) oligonucleotide in which one strand contained both an internal abasic site and a 5′-TAMRA fluorophore whereas the complementary strand contained a 3′-BHQ2 quencher. Later, the same oligonucleotide was suggested by Thomson and co-workers[17] as a substrate for the AP-site cleaving activity of Tdp1 that was discovered by us few years ago.[9] However, we have previously shown that AP-sites inside short (12 and 17 nt) single-stranded (ss) and ds DNA substrates were resistant to Tdp1 cleavage.[10] At the same time, the ability of Tdp1 to remove a variety of quenchers from the 3′-end of DNA has been widely reported, and specific and quantitative real-time measurements of Tdp1-activity using an optical biosensor based on the fluorophore-quencher model have been performed not only in the case of a purified sample of human Tdp1, but also in crude cellular extracts.[16] In light of the above, we thought it is very likely that Thomson and coauthors[17] have in fact monitored the 3′-phosphodiesterase activity of Tdp1 (Figure 1A) instead of the AP-site cleaving activity (Figure 1B). The results we present in this paper support this conclusion. Therefore, no real-time fluorescence-based assay is as yet available that would be able to quantify the AP-site cleaving activity of Tdp1 independently from the activity of APE1 and reliably excluding any other potential phosphodiesterase activity of Tdp1 such as the 3′-quencher removal.

We have shown previously that Tdp1 hydrolyzes the phosphodiester bond on the 5′-side of the natural AP-site and its artificial equivalents such as a common AP-site mimic (2*R*,3*S*)-3-hydroxy-2-hydroxymethyltetrahydrofuran (F), as well as non-nucleotidic insertions such as 1,10-decanediol and diethylene glycol.[11] The cleaving activity of Tdp1 was assessed using a radiolabeled oligonucleotide substrate containing an AP-site or its equivalent and subsequent quantification of the products of cleavage reaction via denaturing polyacrylamide gel electrophoresis (PAGE) and autoradiography.[9−11] This approach is not suitable for real-time quantification of the AP-site cleaving activity of Tdp1. This prompted us to search for alternative assay formats.

We would like to describe herein a new oligonucleotide-based fluorescence assay for a real-time measurement of Tdp1-mediated AP-site cleaving activity, which is based on our previously obtained data.[11] The Tdp1 substrate was a single-stranded oligonucleotide containing an internal AP-site mimic, a 5′-FAM fluorophore, and a 3′-BHQ1 quencher. To prevent unwanted Tdp1-mediated cleavage of the latter, the quencher was attached via a nondigestible 3′-phosphodiester group analogue. The analogue we have used herein is a new tetramethyl phosphoryl guanidine (Tmg) phosphodiester group substitute (see Figure 2 for the structure), which was

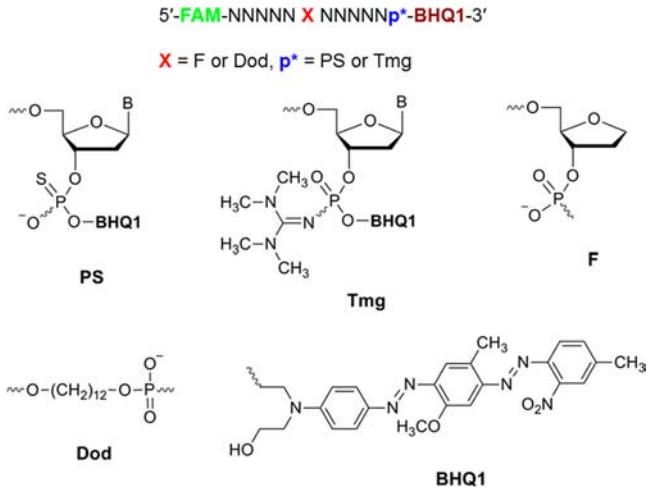

Figure 2. Chemical modifications used in the study. B − nucleobase; BHQ1 − Black Hole Quencher 1; FAM − 5(6)-carboxyfluorescein label; F − (2*R*,3*S*)-3-hydroxy-2-hydroxymethyltetrahydrofuranyl phosphate; Dod − 1,12-dodecanediol phosphate; Tmg − tetramethyl phosphoryl guanidine group; PS − phosphorothioate group.

discovered recently in our Institute[20] and found to be resistant to 3′-phosphodiesterase activity of Tdp1 (Figure 3). As an AP-site mimic we have used an artificial AP-site equivalent 1,12-dodecanediol (Dod) (see Figure 2 for the structure) in the sixth position of a 16 nt oligonucleotide (FAM-Dod^6-Tmg-BHQ) (Table 1). We have shown previously that those synthetic AP-site analogues were suitable as substrates for Tdp1.[11] Also, we have observed previously that Tdp1, unlike APE1, much more efficiently cleaves AP-sites located in the ss DNA regions or those opposite to bulky DNA lesions in the regular DNA duplexes.[10]

We have also designed a DNA duplex with an AP-site analogue tetrahydrofuran (F) in the sixth position of a 16 nt 5′-FAM-labeled strand (FAM-F^6 in Table 1) and a comple-

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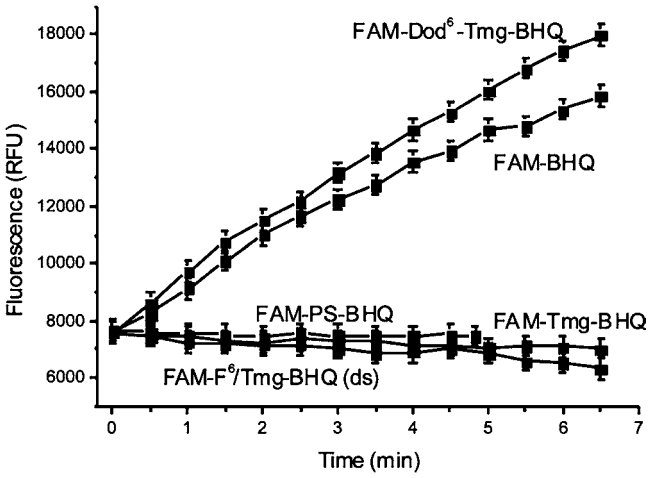

Figure 3. Evaluation of new oligonucleotide probes for real-time measurement of Tdp1 cleavage activity. Fluorescence signal as a function of time for different DNA substrates. Concentrations: 100 nM DNA substrate, 2 nM Tdp1. RFU – relative fluorescence units.

mentary strand containing a 3′-BHQ1 quencher attached via a nondigestible Tmg linkage (Tmg-BHQ in Table 1). This DNA probe was very similar to that used by Thomson et al.[17] except that in ours the quencher was connected by a noncleavable group. As a control we have used a DNA substrate that has had no AP-site and a natural phosphodiester group between the 3′-terminal nucleoside and a BHQ1 quencher (FAM-BHQ). A DNA substrate with a phosphorothioate (PS) linkage (see Figure 2 for the structure) between the 3′-nucleoside and a 3′-BHQ1 quencher (FAM-PS-BHQ) was used as another control, as Tdp1 is known to be incapable to cleave phosphorothioate bonds.[21] Also, we have used another DNA substrate without an AP-site but with a noncleavable Tmg linkage between the 3′-nucleoside and a BHQ1 quencher (FAM-Tmg-BHQ in Table 1) to watch for any 3′-phosphodiesterase activity of Tdp1. The fluorophore and quencher in those ss DNA substrates were positioned in close proximity, and the emission of the fluorophore was thus efficiently suppressed. When Tdp1 cuts at either the AP-site or at the 3′-BHQ1 quencher or at both positions at the same time, those enzymatic reactions may then be monitored by an increase in fluorescence with excitation at 485 nm and emission at 520 nm. As shown in Figure 3, Tdp1 was able to cleave only two DNA substrates: FAM-BHQ through its 3′-phosphodiesterase activity and FAM-Dod^6-Tmg-BHQ using its internal AP-site cleaving activity similarly to what was found previously.[11] As may have been expected, neither FAM-PS-BHQ nor FAM-Tmg-BHQ substrates have been hydrolyzed by Tdp1. The duplex FAM-F^6/Tmg-BHQ was not processed by Tdp1 either. Thus, our results contradict those of Thomson et al.[17] on the AP-site cleaving activity of Tdp1 in a short ds DNA. Therefore, we think that in the referred work[17] the 3′-BHQ group removal by Tdp1 was detected and not the AP-site cleavage.

When the position of the F group was shifted to the 12th position of the 16 nt ss DNA substrate (FAM-F^{12}-Tmg-BHQ), hydrolysis of this substrate by Tdp1 was observed (Figure 4A). This result was confirmed by separation of the cleavage products by gel electrophoresis (Figure 4B). Thus, Tdp1 is more active in the cleavage of an ss DNA containing the F group in the 12th position from the 5′-end (Figure 4B, lane 3) but does not cleave the same group in the sixth position from the 5′-end (Figure 4B, lane 8). We can observe in this lane only the 15 nt oligonucleotide which could be ascribed to the removal of the 3′-terminal nucleoside via the nucleosidase activity of Tdp1.[21] APE1 is known to hydrolyze preferably the AP-site or its synthetic analogue located in one strand of ds DNA.[9] Accordingly, we did not detect any cleavage at the F group in either 6th or 12th position from the 5′-end of the substrate using APE1 (Figure 4B, lanes 2 and 7, respectively).

In this study we have additionally used two mutants of Tdp1 in which specific histidine residues have been replaced. In one of the mutants H493R His 493 was replaced with Arg. This mutation is responsible for an autosomal recessive neurodegenerative disease, spinocerebellar ataxia with axonal neuropathy (SCAN1).[3] In the other mutant H263A His 263 was replaced with Ala. Using ss DNA substrates containing the F group in either 6th or 12th position from the 5′-end, we have shown that both mutants show no AP-site cleaving activity obtained for the WT Tdp1 in the case of FAM-F^{12}-Tmg-BHQ substrate (Figure 4B).

Interestingly, when a Tmg linkage between the 3′-nucleoside and a quencher was replaced by a PS linkage, Tdp1 was unable to cleave at the F group in the substrate FAM-F^{12}-PS-BHQ even when concentration of Tdp1 in the reaction mixture was increased up to 300 nM (Figure 4A). It is possible that Tdp1

Table 1. Designations and Sequences of Oligonucleotide Substrates

no.	designation	sequence, 5′ to 3′[a]	ESI MS, Da	
			calcd	found
1	FAM-Tmg-BHQ	FAM-GGAAGACCCTGACGTTp*-BHQ	6131.44	6131.91
2	FAM-PS-BHQ	FAM-GGAAGACCCTGACGTTp$_S$-BHQ	6348.69	6345.10
3	FAM-F^{12}-Tmg-BHQ	FAM-GGAAGACCCTGFCGTTp*-BHQ	5998.33	5998.71
4	FAM-F^{12}-PS-BHQ	FAM-GGAAGACCCTGFCGTTp$_S$-BHQ	5913.24	5909.91
5	FAM-Dod^6-Tmg-BHQ	FAM-GGAAGDodCCCTGACGTTp*-BHQ	6078.50	6084.38
6	FAM-Dod-Tmg-BHQ	FAM-GGAAGADodTCTTCCp*-BHQ	5134.93	5129.52
7	FAM-Dod-PS-BHQ	FAM-GGAAGADodTCTTCCp$_S$-BHQ	5049.84	5046.72
8	FAM-BHQ	FAM-GGAAGACCCTGACGTT-BHQ	nd[b]	nd
9	FAM-F^6	FAM-GGAAGFCCCTGACGTT	nd[b]	nd
10	Tmg-BHQ	AACGTCAGGGTCTTCCp*-BHQ[c]	5510.92	5509.50

[a]Template sequence 5′-d(GGAAGACCCTGACGTT). [b]Obtained from a commercial supplier. [c]Complementary sequence FAM-F^6; BHQ – Black Hole Quencher 1; FAM – 5(6)-carboxyfluorescein label; F – (2*R*, 3*S*)-3-hydroxy-2-hydroxymethyltetrahydrofuranyl phosphate; Dod – 1,12-dodecanediol phosphate; p* – position of a tetramethyl phosphoryl guanidine (Tmg) group; p$_S$ – position of a phosphorothioate (PS) group (see Figure 3 for the structures).

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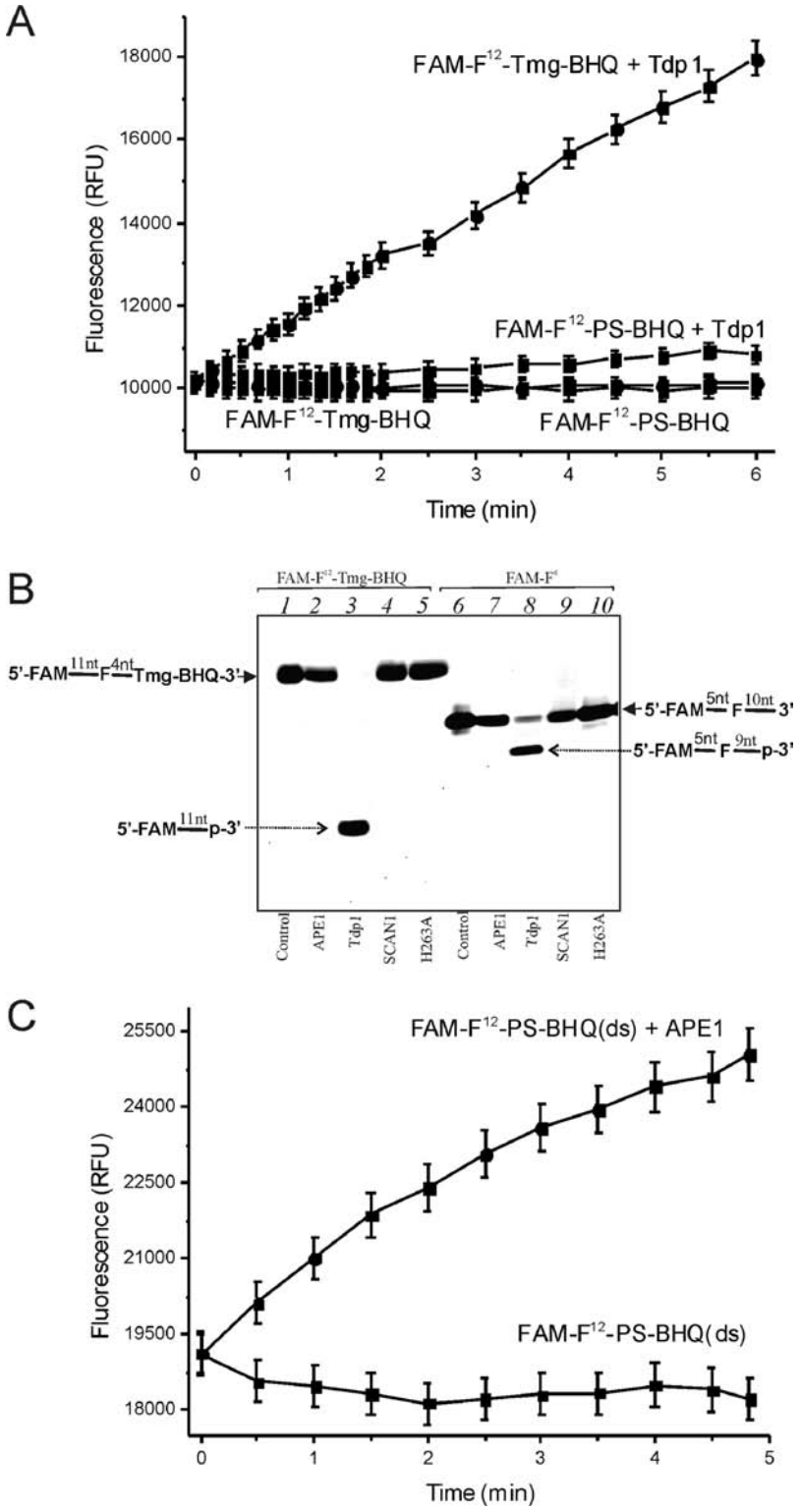

Figure 4. Efficiency of Tdp1 cleavage with 16-mer DNA substrates depending on the abasic site mimic F position. (A) Fluorescence signal as a function of time with DNA substrates containing the F residue in the 12th position. Concentrations: 250 nM DNA substrate, 100 nM Tdp1 for FAM-F[12]-Tmg-BHQ substrate and 300 nM Tdp1 for FAM-F[12]-PS-BHQ substrate. RFU − relative fluorescence units. (B) Electrophoretic analyses of Tdp1 (100 nM) hydrolysis products with DNA substrates (100 nM) containing the F residue in the 6th or 12th position. The products of hydrolysis were separated by 15% polyacrylamide gel electrophoresis under denaturing conditions and visualized by fluorometry. (C) APE1 hydrolysis of the PS dsDNA substrate, which was obtained by annealing of FAM-F[12]-PS-BHQ with the complementary 16-mer oligonucleotide 5′-d(AACGTCAGGGTCTTCC). Concentrations: 100 nM DNA substrate, 2 nM APE1. RFU − relative fluorescence units.

activity is noticeably decreased in the presence of a PS linkage. At the same time, APE1 cleaves at the F group in the ds DNA containing a 3′-BHQ1 quencher attached via a PS linkage (Figure 4C). Thus, the fluorescence assay developed by us is

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suitable for screening for an AP-site cleaving activity of Tdp1 that would be clearly distinguishable from that of APE1.

These results enabled us to design a new DNA biosensor capable of detecting the AP-site cleaving activity of a potential anticancer drug target Tdp1 in a simple, high-throughput, and real-time format. To improve sensitivity and selectivity of the assay we have used a hairpin DNA biosensor containing a Dod moiety as an artificial AP-site mimic and a 3′-quencher (BHQ1) and 5′-fluorophore pair at a blunt end. To avoid the removal of the quencher by the 3′-phosphodiesterase activity of the enzyme and its seal on the PS linkage, the BHQ1 residue was attached to the 3′-end through a tetramethyl phosphoryl guanidine (Tmg) linkage, the presence of which does not inhibit the AP-site cleaving activity of Tdp1. The DNA sequence was designed with internal complementary sequences connected by a flexible Dod loop (Figure 5). Such a hairpin

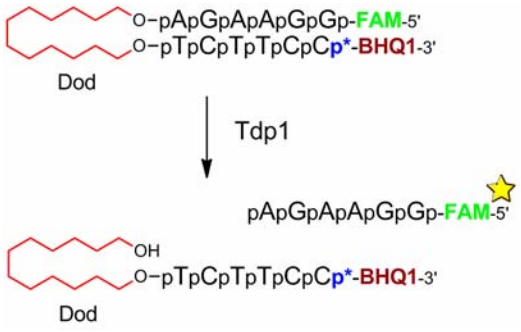

Figure 5. Design of a hairpin oligonucleotide biosensor with a 1,12-dodecanediol AP-site mimic for a real-time fluorophore-quencher assay of Tdp1-mediated AP-site cleavage. p* is either a Tmg or a PS group.

DNA biosensor was hydrolyzed by Tdp1 only; APE1 was not able to cleave at the Dod moiety in this looped structure (Figure 6A). However, it was shown that APE1 was able to cleave quite efficiently at a similar 1,10-decanediol bridge in the middle of one strand of a ds DNA.[22] A looped DNA substrate with a Dod moiety and a 3′-BHQ1 attached to DNA through a PS linkage was used to examine the AP-site processing by Tdp1 (Figure 6B). As we anticipated, the presence of a PS linkage in this looped DNA substrate decelerated the AP-site cleaving activity of Tdp1. Using fluorescent substrate titration data, the initial rates were plotted as a function of the substrate concentration (Figure 7) and the kinetic parameters of the AP-site cleaving reaction of Tdp1 were estimated: k_{cat} = 1.3 ± 0.2 min^{-1}, K_m = 301 ± 74 nM for the FAM-Dod-PS-BHQ substrate; k_{cat} = 4.1 ± 0.5 min^{-1}, K_m = 332 ± 71 nM for the FAM-Dod-Tmg-BHQ substrate; and k_{cat} = 0.10 ± 0.01 min^{-1}, K_m = 161 ± 33 nM for FAM-F^{12}–Tmg-BHQ. These data could be interpreted as evidence that Tdp1 cuts more efficiently at the Dod moiety in the looped substrates than at the F moiety in the 12th position of an ss DNA.

To provide additional evidence for high efficiency of the FAM-Dod-Tmg-BHQ probe for monitoring the AP-site cleaving activity of Tdp1, we have performed a kinetic analysis using this probe (Figure 8A) in comparison with FAM-F^{12}-Tmg-BHQ substrate (Figure 8B) in the wide range of Tdp1 concentration. The data obtained have demonstrated that the looped FAM-Dod-Tmg-BHQ probe is ~50 time more sensitive to Tdp1 then the linear FAM-F^{12}-Tmg-BHQ (Figure 8C).

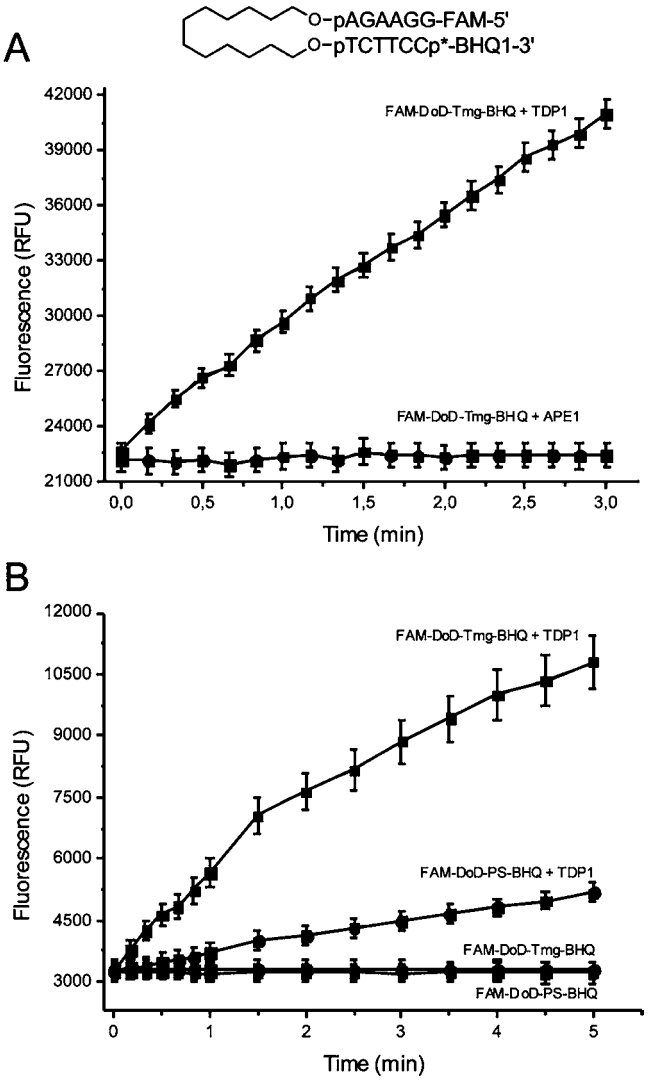

Figure 6. Fluorogenic Tdp1 AP-site cleavage assay. (A) Design and evaluation of a new hairpin probe for a real-time measurement of the AP-site cleaving activity of Tdp1 or APE1. Concentrations: 100 nM substrate, 5 nM Tdp1 and 10 nM APE1. RFU – relative fluorescence units. (B) Comparative analyses of Tdp1 cleaving activity on a hairpin DNA substrate containing either PS or Tmg linkage. Concentrations: 170 nM substrate, 10 nM Tdp1. RFU – relative fluorescence units.

Finally, we have demonstrated that FAM-Dod-Tmg-BHQ probe is resistant both to exo- and endonuclease digestion under conditions when Tdp1 is able to cleave this substrate (Figure 9). Therefore, this DNA probe can be used for specific detection of AP-site cleavage by Tdp1 in the presence of contaminating nucleases.

Thus, we have developed a highly specific and sensitive real-time quantification method for the AP-site cleaving activity of Tdp1 using a short hairpin oligonucleotide having a 5′-fluorophore and a 3′-quencher with a 1,12-dodecanediol loop as an artificial AP-site mimic. Our approach would allow one to clearly distinguish the specific AP-site cleaving activity of Tdp1 from that of APE1, which is not provided by other fluorescence-based Tdp1 activity assays.[16,18] The 3′-phosphodiesterase activity of Tdp1 that may be normally expected to remove the quencher from the 3′-end of DNA[17] was suppressed in our approach by introducing a new noncleavable phosphodiester group mimic tetramethyl phosphoryl guanidine

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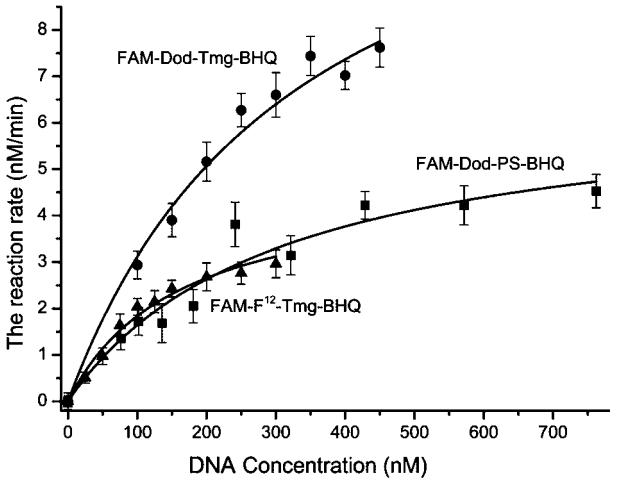

Figure 7. Kinetic curves of the AP-site cleavage reaction catalyzed by Tdp1. The initial rates were determined from fluorescence measurement data. Substrate concentrations were varied in the range of 0−450 nM for FAM-Dod-Tmg-BHQ with 3.3 nM Tdp1; 0−750 nM for FAM-Dod-PS-BHQ with 5 nM Tdp1; 0−300 nM for FAM-F[12]-Tmg-BHQ with 50 nM Tdp1.

(Tmg) group rather than a known phosphorothioate group as the latter decreased the rate of AP-site cleavage by the enzyme. The hairpin oligonucleotide described herein would be much simpler and less expensive to prepare, and there are no requirements for additional reagents apart from the substrate and the enzyme unlike in the other formats discussed.[23,24] That makes the fluorescence-based Tdp1-mediated AP-site cleavage assay described herein potentially very effective, particularly for screening large libraries of compounds for Tdp1 inhibitors.

EXPERIMENTAL PROCEDURES

Recombinant wild-type (WT) Tdp1 and mutant human Tdp1 proteins (SCAN1 and H263A) were purified to homogeneity by chromatography on Ni-chelating resin and cellulose phosphate P11 as described.[10,25] A recombinant purified APE1 was a generous gift from Dr. Svetlana Khodyreva (ICBFM, Novosibirsk). A recombinant *E. coli* exonuclease III (Exo III) was from SibEnzyme (Novosibirsk), pancreatic deoxyribonuclease I (DNase I) was from Sigma.

Oligonucleotides were synthesized on a Biosset ASM-800 automated DNA synthesizer (Russia) on 200 nmol scale using *β*-cyanoethyl phosphoramidite chemistry. Tetrahydrofuranyl group (F) was introduced via dSpacer phosphoramidite from Glen Research (USA). 1,12-Dodecanediol residue (Dod) was introduced via the corresponding dimethoxytrityl-1,12-dodecanediol phosphoramidite prepared as described previously.[26] 5(6)-FAM phosphoramidite for 5′-fluorescein 5(6)-amide (FAM) labeling and a solid support 3′-BHQ-1 CPG for attachment of a Black Hole Quencher BHQ-1 residue were a generous gift of Dr. Vladimir Ryabinin (ICBFM, Novosibirsk). Oligonucleotides FAM-GGAAGACCCTGACGTT-BHQ1 (FAM-BHQ) and FAM-GGAAGFCCCTGACGTT (FAM-F[6]) without either phosphorothioate (PS) or Tmg blocking groups were obtained from a commercial supplier via the Laboratory of Medicinal Chemistry (ICBFM, Novosibirsk). PS linkages were introduced with Sulfurising reagent II (3-(dimethylaminomethylidene)amino-3*H*-1,2,4-dithiazole-3-thione, DDTT) from Glen Research (USA) according to the manufacturer's protocol. Incorporation of a Tmg group was performed as described.[20] After the completion of solid-phase synthesis, polymer support from the column was transferred to a plastic tube and treated with 200 *μ*L of concentrated (ca. 25%) aqueous ammonia solution per 5 mg of support at 55 °C

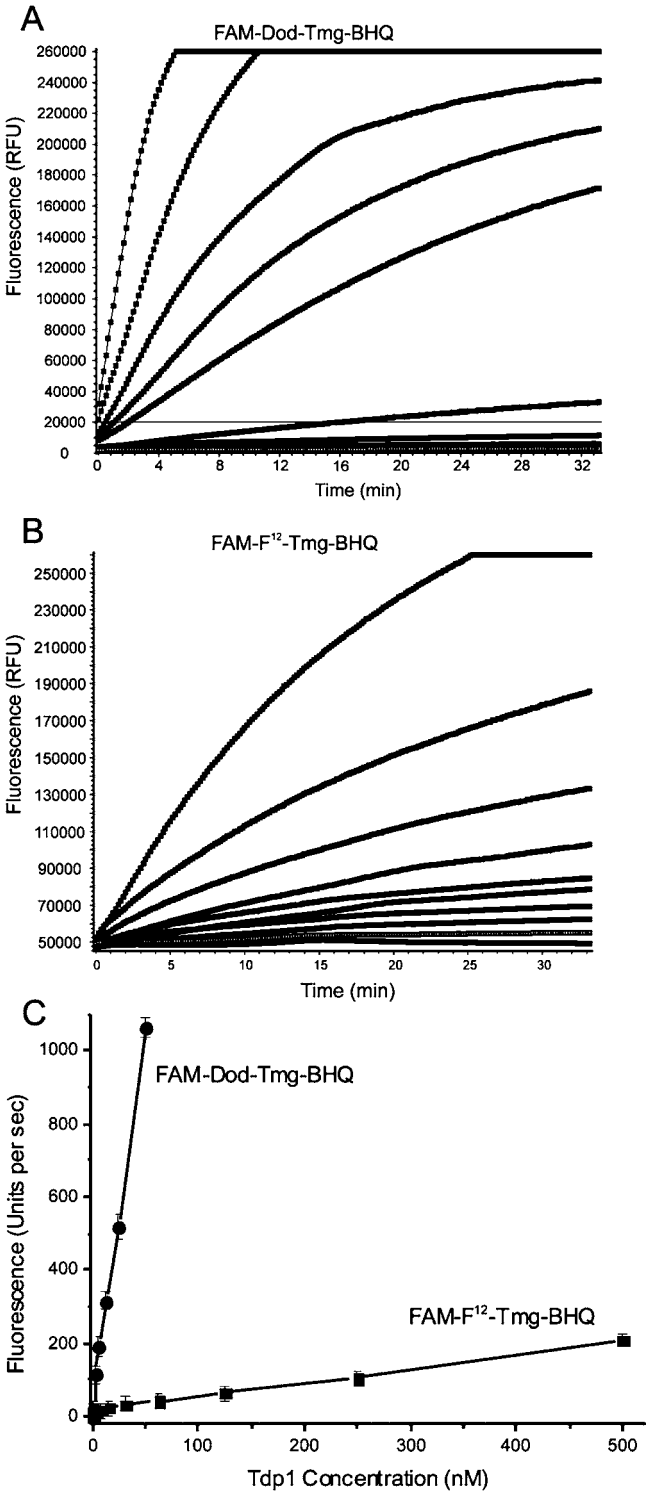

Figure 8. Comparative analyses of Tdp1 cleaving activity on the Dod containing hairpin and F[12] containing linear DNA substrates. (A) Tdp1 titration followed in kinetic mode with FAM-Dod-Tmg-BHQ and (B) FAM-F[12]-Tmg-BHQ. (C) Kinetic data from panels A and B were plotted as a function of Tdp1 concentration. Concentrations: 100 nM substrate, 0−50 nM (A) or 0−500 nM (B) Tdp1. RFU − relative fluorescence units.

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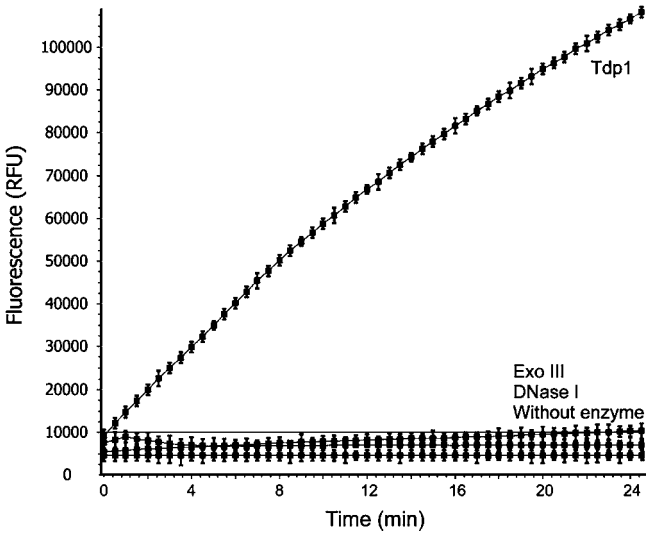

Figure 9. Fluorogenic nuclease digestion assay. Fluorescence signal as a function of time with FAM-Dod-Tmg-BHQ probe in the presence of DNase I, Exo III, or Tdp1. Concentrations: 100 nM substrate, 20 nM Tdp1, 50 units/μL Exo III, 0.5 mg/mL DNase I.

for 16 h. After deprotection the supernatant was evaporated in vacuo using a ThermoFisher SpeedVac concentrator (USA), 400 μL of 20 mM triethylammonium acetate, pH 7 was added, and support removed by centrifugation.

Oligonucleotides were purified by reverse-phased (RP) HPLC on an Agilent 1200 series HPLC system (USA) equipped with a Zorbax SB-C18 (5 μm) column (4.6 × 150 mm) using a gradient of acetonitrile from 0% to 40% in 0.02 M triethylammonium acetate, pH 7 for 30 min, flow rate 2 mL/min. Denaturing gel electrophoresis in 20% polyacrylamide gel (PAGE) was used to check the purity of oligonucleotides with band visualization by staining with Stains-All (Sigma). Molecular masses of modified oligonucleotides were confirmed by electrospray ionization (ESI) mass spectra recorded on an Agilent G6410A LC-MS/MS triple quadrupole ESI mass spectrometer (USA) in the MS scan mode with negative ion detection. The oligonucleotides were dissolved to 0.1 mM concentration in 20 mM triethylammonium acetate containing 60% acetonitrile for direct injection (10 μL). Elution was by 80% acetonitrile in isocratic mode, flow rate 0.1 mL/min. Default parameters for ESI and MS were used for all the experiments: nebulizer gas pressure was 30 psi (207 kPa), drying gas (nitrogen) flow rate was 9 L/min and temperature 340 °C, capillary voltage was 4000 V, detected mass range was from m/z 105 to 1600. Molecular masses of oligonucleotides were obtained from the spectra manually using the formula $z_2 = /(m/z_1 + /1)/(m/z_1 /-m/z_2)$.

Structures of the chemical modifications used in this study are depicted in Figure 3. Sequences and ESI MS data of the oligonucleotides are given in Table 1. Complementary oligodeoxyribonucleotides were annealed in equimolar amounts by heating their solution to 95 °C for 3 min followed by slow cooling to ambient temperature.

Fluorescence Measurements. Reaction mixtures (100 μL) contained 50 mM Tris-HCl, pH 8.0, 1 mM DTT, 50 mM NaCl, 100 nM DNA substrate, and given amounts of Tdp1 or other enzymes. For APE1, 5 mM $MgCl_2$ was added. The assay was designed to be performed in 96-well black plates. After addition of Tdp1 the reactions were incubated at a constant temperature of 25 °C for 15 min in a microplate reader for a real-time measurement of their fluorescence every 30 s. Fluorescence measurements and data analyses were performed with a POLARstar OPTIMA or CLARIOstar multifunctional microplate reader and MARS Data Analysis Software (BMG LABTECH GmbH, Germany). The enzyme kinetic parameters were calculated from reaction rate vs concentration data according to Michaelis−Menten equation and hyperbolic fit to produce V_{max} and K_m. Excitation of fluorescent probes was performed at 485 nm (485BP1 filter) with fluorescence detection at 520 nm (EM520 filter). Each experiment was repeated at least three times.

AP-Site Cleavage Assays. The standard reaction mixtures (10 μL) contained 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 100 nM for DNA substrate and 100 nM Tdp1 or APE1. After addition of Tdp1 the reaction mixtures were incubated at 37 °C for 30 min. Then reactions were terminated by adding a solution of Xylene Cyanol and Bromophenol Blue dyes in formamide, and the mixtures were heated for 3 min at 90 °C. The products were analyzed by electrophoresis in 15% polyacrylamide gel with 8 M urea.[27] The products were visualized using Molecular Imager (Bio-Rad) and Quantity One software.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AP-site, apurinic/apyrimidinic site; Dod, 1,12-dodecanediol; Tdp1, tyrosyl-DNA phosphodiesterase 1; Tmg, tetramethyl phosphoryl guanidine

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