Systematic Probing of the Sequence Selectivity of Exonuclease III with a Photosensitization Colorimetric Assay

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Supporting Information

ABSTRACT: Exonuclease III (Exo III) is an important enzymatic tool that is being widely used in molecular biology, biotechnology, and bioassay development. Exo III prefers to cleave double-stranded DNA (dsDNA) with blunt and recessed 3′-termini rather than their protruding counterparts. While it has been accepted that a short 3′-overhang (e.g., >4 nt) is necessary to protect a dsDNA from Exo III cleavage, critical roles of the length and sequence of this 3′-overhang remain unexplored. Herein, we develop a novel light-induced colorimetric assay allowing the systematic probe of the sequence selectivity of Exo III in a rapid and high-throughput manner. Our finding that Exo III is highly specific to 3′-overhang in terms of both length and sequence will be valuable for guiding the design of bioassays and DNA manipulating tools mediated by Exo III.

INTRODUCTION

Exonuclease III (Exo III) that catalyzes the stepwise removal of mononucleotides from 3′-hydroxyl termini of a double-stranded DNA (dsDNA) is being widely used as an enzymatic tool for diverse biological and biotechnological applications.1−4 It has also been adopted for developing sensitive analytical assays for biologically and clinically important molecules, such as nucleic acids, proteins, and small molecules.5−8 When applying Exo III to bioassay development, it is critical to ensure the target-specific dsDNA cleavage, which relies primarily on the high substrate selectivity of Exo III to dsDNA over single-stranded DNA (ssDNA).5,8,21−25 A dsDNA with 3′-protruding termini may also resist Exo III cleavage, but such a resistance depends on the length of the 3′-overhang.26 Brutlag and Kornberg observed that dsDNA with up to three mismatched terminal nucleotides at 3′-termini could be hydrolyzed by Exo III, whereas the terminal mismatches of more than 4 nt were resistant to the cleavage.27 As such, a frequently used rule of thumb to protect dsDNA from the Exo III cleavage for bioassay development is to use a 3′-overhang with a length of more than 4 nt.24,28 However, the substrate selectivity of Exo III toward the length of the 3′-overhang has never been systematically probed. It is also unclear if the substrate selectivity of Exo III also depends on the sequences of the 3′-overhang.29 To meet this knowledge gap and guide the better design of Exo III-assisted assays, it is critical to systematically examine the selectivity of Exo III to 3′-overhangs of the dsDNA substrates.

Classic methods for studying the catalytic activity of Exo III,14,32 such as radioisotope tracing,25 and gel electrophoresis,26 are tedious, time-consuming, and unable to monitor the cleavage reaction in real time. The use of radioisotopes (3H or γ-[32P] ATP-labeled DNA) is not user-friendly. Herein, we develop a simple and rapid light-induced...
photosensitization colorimetric assay is capable of systematically probing the sequence selectivity of Exo III in a high-throughput time-resolved manner (Figure 1A). This assay is developed based on our previous observation that the widely used DNA intercalating dye, SYBR Green I (SG), could serve as a type II photosensitizer upon intercalation to dsDNA and trigger the conversion of a colorless 3′,3′,5′,5′-tetramethylbenzidine (TMB) substrate to its blue oxidative product (oxTMB). As this light-induced reaction is simple, sensitive, and highly selective, we harness it as a colorimetric readout for measuring the activity of Exo III.

**RESULTS AND DISCUSSION**

**Photosensitization Colorimetric Assay for Exo III Activity Probing.** The light-induced photosensitization colorimetric assay capable of probing the activity of Exo III is illustrated in Figure 1A. A dsDNA can be quantified colorimetrically using SG as a photosensitizer and TMB as a chromogenic substrate. Upon light irradiation, the SG intercalated in the dsDNA triggers the production of a highly reactive singlet oxygen (1O2) that oxidizes the colorless TMB into blue oxTMB. We have shown previously that the sensitivity of our photosensitization colorimetric assay is comparable with the fluorescence spectroscopy.

**Figure 1.** (A) Schematic illustration of the detection of Exo III activity based on photosensitization colorimetric assay system. (B) Absorbance profile of TMB upon photosensitized oxidation in the absence and presence of Exo III. (C) Effects of different DNA structures on enzyme activities.

The cleavage efficiency is defined as

\[
\text{cleavage efficiency} = \frac{A_{\text{hairpin}} - A_{\text{hairpin+Exo III}}}{A_{\text{hairpin}} - A_{\text{loop} - 5'}}
\]

where \(A_{\text{hairpin}}\) and \(A_{\text{hairpin+Exo III}}\) are the absorbances of oxTMB in the presence of the hairpin substrate and the hairpin substrate after the Exo III cleavage, respectively. \(A_{\text{loop} - 5'}\) is defined as the absorbance of oxTMB in the presence of the green part ssDNA (Figure 2A, 5′-end to the loop part, Table S1), which was subtracted from \(A_{\text{hairpin}}\) to correct for the assay background.

**Figure 2.** (A) Substrate selectivity of Exo III toward dsDNA with 3′-recessed, blunt, and protruding ends. (B) Gel-electrophoresis results of the Exo III cleavage; (C) activity of Exo III toward 3′-protruding ends of different overhang lengths. The detailed sequences used here are given in Table S1. Error bars represent the standard deviation from triplicate analyses.

To confirm that hairpin substrates are suitable for probing the activities of Exo III, we first measured the activity of Exo III against three hairpin substrates bearing 3′-recessed, blunt, and protruding ends. The absorbances of oxTMB were measured in the presence of hairpin substrates and hairpin substrates after the Exo III cleavage using the colorimetric assay (Figure 1B). Using this assay, we were able to probe the Exo III activity at varying assay conditions including Mg2+, Mg2+, temperature, and ethylenediaminetetraacetic acid (EDTA) (Figures S1–S3).

**Confirming the Substrate Selectivity of Exo III Using the Photosensitization Colorimetric Assay.** To investigate the specific roles of the 3′-termini of a dsDNA substrate, we designed a series of hairpin-based substrates containing a 8 nt loop, a 30 bp stem, and varying 3′-termini designs (Table S1). Compared to the dsDNA substrate, the hairpin substrate contains only one 3′-overhang (Figure 2A). The use of hairpin probes as substrates may also help avoid potential stoichiometry variations for preparing the dsDNA duplex.
found that 10 of all 16 sequences in the weakly resistant group strongly resistant with cleavage efficiency of the hairpin substrates. Based on the observed cleavage III was highly dependent on the sequence of the 3 nt overhang unique groups: weakly resistant to Exo III with cleavage efficiency, moderately resistant to Exo III with cleavage efficiency, and strongly resistant to Exo III with cleavage efficiency.

We next investigated the selectivity of Exo III to the sequence of the 3 nt overhangs because of the interesting observation mentioned above. We then tested the activity of Exo III against the hairpin substrates with varying overhang lengths from 1 to 8 nt at the protruding-3′-termini (Figure 2C, with random 3′-overhang sequences; Table S1). Consistent with the previous report by Brutlag and Kornberg,27 dsDNA was found to efficiently resist the Exo III cleavage when the 3′-overhang is 4 nt or longer, while Exo III is fully active when the 3′-overhang is 1 or 2 nt. Interestingly, Exo III became partially active (25% activity) upon a 3′-overhang of 3 nt. All observations were further confirmed using the PAGE analyses (Figure S5). Despite the consistency between the two methods, our assay is simpler, much faster, and more sensitive than PAGE (Figure S6).

**Sequence Selectivity of Exo III on 3 nt 3′-Overhangs.**

We next investigated the selectivity of Exo III to the sequence of 3′-overhang. The length of the overhang was fixed at 3 nt because of the interesting observation mentioned above. Hairpin substrates with possibly 64 combinations of the nucleotide sequences of 3′-overhangs (Table S2) were designed and systematically examined using the light-induced colorimetric assay (Figure 3). We found that the activity of Exo III was highly dependent on the sequence of the 3 nt overhang of the hairpin substrates. Based on the observed cleavage efficiency, we were able to classify the 64 substrates into three unique groups: weakly resistant to Exo III with cleavage efficiencies >20% (Figure 3A), moderately resistant with cleavage efficiencies between 10 and 20% (Figure 3B), and strongly resistant with cleavage efficiencies <10% (Figure 3C).

By carefully examining the sequences in each group, we found that 10 of all 16 sequences in the weakly resistant group terminated with “C” at the 3′ end, while the number of sequences that terminated with “A”, “T”, and “G” were 3, 1, and 1, respectively (Figure 3A). By contrast, only 4 out of 32 sequences were terminated with C in the strong resistance group (Figure 3C) and most sequences in this group ended with G (11) or T (11). Our observation in the sequence selectivity of Exo III (C > A > G > T) is close to the observation by Linxweiler and Hörz that the activity of Exo III to follow the trend C > A = T > G.21 We also performed kinetic analyses for six representative hairpin substrates, where -AAC, -CGG, -CGA, and -GAA were chosen from the weakly resistant group and -TCC and -TTT were picked from the strongly resistant group. The observed kinetics of the Exo III cleavage for the six chosen substrates were consistent with that of the observed groups determined by the end-point measurements (Figure 4).

**Sequence Selectivity of Exo III on Longer 3′-Overhangs.**

We next extended the six chosen 3′-overhangs as mentioned above with additional nucleotides so that the hairpin substrates were of longer 3′-overhangs but terminated with the same 3 nt combination (Table S3). Despite the observation that longer 3′-overhangs were more resistant to the Exo III cleavage, some of the weakly resistant combinations (e.g., -AAC and -CGG) still show a higher level of cleavage than others (Figure 5), suggesting that the observed trend in Figure 3 may be extended to dsDNA substrates with even longer 3′-overhangs. Of note, Linxweiler and Hörz21 concluded that Exo III digestion was impossible for the 4 nt cases. However, the substrate difference may also contribute to such an inconsistency, since a different system with pBR322 fragments generated by restriction endonucleases (overhangs of 4 and 2 nt and blunt ends) as well as synthetic linker fragments was used as a substrate.21 To validate the potential uses of the observed trend for guiding the sequence design of Exo III-mediated bioassays, we

**Figure 3.** Evaluating the sequence selectivity of Exo III toward dsDNA with 3 nt overhangs at the protruding-3′-termini. (A) Group weakly resistant to Exo III hydrolysis; (B) group moderately resistant to Exo III hydrolysis; and (C) group strongly resistant to Exo III hydrolysis. The green and orange dotted lines indicate cleavage efficiencies of 20 and 10%, respectively. Results are averages of six parallel studies with the corresponding standard deviations.
Finally extracted 187 DNA sequences from 150 recently published works that successfully made use of the sequence selectivity of Exo III for bioassay development (Table S4). These DNAs were originally designed to be resistant to the Exo III cleavage. We then classified all literature sequences according to their last three nucleotides on the 3′-overhangs (Figure 6) into three groups. Of which, 72% (134 sequences, group 3) of all literature sequences fall into the strongly resistant group of Exo III shown in Figure 3, 20% (37 sequences, group 2) in the moderately resistant group, and only 8% in the weakly resistant group. This result suggests that the observed trend in our work is highly relevant to the successful sequence designs for Exo III-mediated assays in the past years and thus can potentially be used as a general guide for future assay development.

**CONCLUSIONS**

We have systematically probed the substrate selectivity of Exo III toward the length and sequence of the 3′-overhangs of the dsDNA substrates using a light-induced colorimetric assay. Exo III is one of the most frequently used enzymatic tools for bioassay development. To be target specific, the activity of Exo III has to be sequestered by protecting the dsDNA substrate with a protruding 3′-overhang, which could be eliminated through a target-induced conformation change. Therefore, the design of the 3′-overhang plays a vital role in the development of Exo III-mediated bioassays. However, current design strategies are empirical and require many trial-and-error attempts to ensure an optimal assay performance. Our effort to systematically probe the sequence selectivity of Exo III reveals that both length and sequence of a 3′-overhang play critical roles in protecting the dsDNA from Exo III cleavage. We also established a group of three-letter nucleotide combinations at the 3′-termini, which are highly resistant to the Exo III cleavage. More importantly, our findings are highly consistent with the 187 successful dsDNA substrate designs from 150 recent published works. Therefore, we anticipate our work will also serve as a general guide for designing sequences for Exo III-mediated bioassay development and beyond.

**EXPERIMENTAL SECTION**

**Materials.** 3,3′,5,5′-Tetramethylbenzidine (TMB), citrate, disodium hydrogen phosphate, sodium chloride, and dimethyl sulfoxide were from Aladdin (Shanghai, China). Magnesium chloride hexahydrate, calcium chloride, and hydrochloric acid were purchased from Kelong Reagent Co. (Chengdu, China). SYBR Green I (SG, 10 000×), Exo III, and deoxyribonuclease I (DNase I) were from ThermoFisher Scientific. The concentration of 10 000× SG is calculated to be 19.6 mM according to the research from Vitzthum et al. All the oligonucleotides were provided by Shanghai Sangon Biotech Co. Ltd.
Exo III Assay. To obtain the hairpin structure, all of the oligodeoxynucleotides were dissolved with the hybridization buffer (20 mM Tris–HCl, pH 8.0, 20 mM NaCl) to give the final concentration of 10 μM. Then, the above DNA solution was heated at 90 °C for 10 min and cooled to 25 °C overnight. In a typical Exo III assay, a hairpin DNA substrate (1.6 μM) and Exo III (40 U) were mixed in 100 μL of Tris–HCl buffer (pH 8.0, 10 mM MgCl₂). The mixture was then incubated at 37 °C for the Exo III cleavage, followed by deactivation of Exo III at 80 °C for 10 min according to the user manual for Exo III. After cooling down, the mixture was diluted to 2 mL using citrate buffer (pH 4, 50 mM MgCl₂) containing 3.92 μM 6G and 0.2 mg/mL TMB, incubated for 10 min, and then irradiated using cyan light-emitting diode (3 V, 3 W) for 2 min for developing the colorimetric readout. The absorbance of this mixture was then measured at 655 nm for quantifying the activity of Exo III in terms of cleavage efficiency. For all the measurements, at least three parallels were carried out.

Native Polyacrylamide Gel Electrophoresis (Native-PAGE) Analysis. A 12% native polyacrylamide gel was prepared using 2.0 mL 5X TBE (445 mM Tris×boric acid, 200 mM NaCl) buffer, 3.9 mL distilled water, 4.0 mL 30% acrylamide–bisacrylamide (29:1), 0.1 mL 10% ammonium persulfate, and 6.0 μL Tetramethylethylenediamine. A mixture of 10 μL of Exo III reaction product and 2.0 μL of 6X loading buffer was loaded to the PAGE gel and run in 1X TBE buffer at 140 V for 60 min. The gel was then stained with 4S GelRed (Shanghai Sangon Biotech Co. Ltd., China) for 5 min. Photographs were then taken using a C300 Gel imager (Azure Biosystem).

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01560.

Experimental results for optimization of the Exo III assay, DNA sequences, and the reference DNA sequences in Figure 6 (PDF)

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Notes
The authors declare no competing financial interest.

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