1-1-2013

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Published online 27 February 2013

Nucleic Acids Research, 2013, Vol. 41, No. 8 ***4613–4627***
doi:10.1093/nar/gkt117

Translocation of *Saccharomyces cerevisiae* Pif1 helicase monomers on single-stranded DNA

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Received November 19, 2012; Revised January 30, 2013; Accepted February 1, 2013

ABSTRACT

In *Saccharomyces cerevisiae* Pif1 participates in a wide variety of DNA metabolic pathways both in the nucleus and in mitochondria. The ability of Pif1 to hydrolyse ATP and catalyse unwinding of duplex nucleic acid is proposed to be at the core of its functions. We recently showed that upon binding to DNA Pif1 dimerizes and we proposed that a dimer of Pif1 might be the species poised to catalysed DNA unwinding. In this work we show that monomers of Pif1 are able to translocate on single-stranded DNA with 5′ to 3′ directionality. We provide evidence that the translocation activity of Pif1 could be used in activities other than unwinding, possibly to displace proteins from ssDNA. Moreover, we show that monomers of Pif1 retain some unwinding activity although a dimer is clearly a better helicase, suggesting that regulation of the oligomeric state of Pif1 could play a role in its functioning as a helicase or a translocase. Finally, although we show that Pif1 can translocate on ssDNA, the translocation profiles suggest the presence on ssDNA of two populations of Pif1, both able to translocate with 5′ to 3′ directionality.

INTRODUCTION

Saccharomyces cerevisiae Pif1 is a 5′ to 3′ helicase that belongs to the SF1 family of helicases and possibly the prototype member of a new subfamily (1 4). Genetic and biochemical studies indicate that Pif1 participates in a wide range of DNA processing steps, both in the nucleus and in mitochondria (1,4 9). In mitochondria Pif1 plays a role in mitochondrial (mt)DNA repair and recombination and mtDNA maintenance (3,4,10,11). The nuclear form of Pif1 has both telomeric and non-telomeric functions. Pif1 functions in replication fork progression through ribosomal DNA, where it promotes efficient progression of the replication fork at Replication Fork Barrier (RFB) sites (8). Also, Pif1 is involved in Okazaki fragment processing where in conjunction with the Dna2 helicase/nuclease it helps to process long flaps that cannot be directly cleaved by the FEN1 nuclease (7,12 14). A potential role of Pif1 in preventing genomic instability via destabilization of G4-DNA structures (i.e. G-quadruplex) has recently been proposed (15 18).

At telomeres Pif1 acts as a negative regulator, inhibiting telomere elongation and *de novo* telomere addition (6,9,19 21). It has been shown that Pif1 displaces the telomerase from the telomeric end, providing biochemical evidence for the inhibitory effect of Pif1 on the activity of the telomerase (6). Moreover, biochemical studies strongly suggest that Pif1 unwinds RNA DNA heteroduplexes with higher efficiency than dsDNA (22). Therefore, it has been proposed that the unwinding by Pif1 of the RNA DNA heteroduplex formed by the telomeric TLC1 RNA of the telomerase and the ssDNA at telomeres leads to telomerase displacement (2,6,22). Although it is clear that the ATPase activity of Pif1 is required for displacement of the telomerase (6,9), whether Pif1 displaces the telomerase using exclusively its unwinding activity is not well-established.

In addition to its effect on telomere length, deletion of PIF1 leads to an even more striking effect on the *de novo* telomere addition pathway to double-stranded DNA breaks (DSBs) (19 21). Although it is apparent that the activity of Pif1 on *de novo* telomere addition is via a direct effect on the telomerase (19), whether the mechanism of telomerase inhibition by Pif1 at either telomeres or DSBs is the same is not known.

One common feature of all these pathways is that all of the activities of Pif1 rely on its ability to bind to DNA and hydrolyse ATP. We recently determined the mode of Pif1 binding to different DNA substrates (23). Although Pif1 is a monomer in solution, we showed that Pif1 dimerizes upon binding to DNA (23). DNA-induced dimerization of Pif1 is observed on ssDNA as well as tailed- and forked-dsDNA substrates. A dimer of Pif1 also forms on DNA unwinding substrates in the presence of saturating concentrations of non-hydrolysable ATP analogs (23). These observations suggest that a dimeric form of

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the enzyme might constitute the pre-initiation complex required for unwinding activity. Whether a dimer or monomer of Pif1 is sufficient for DNA unwinding activity remains to be explored. Also, intrinsic to its ability to unwind dsDNA a helicase must be able to translocate along at least one strand of the duplex (24,25). In the case where translocation can be decoupled from unwinding, a helicase will also be able to translocate along ssDNA. Therefore, it is possible that regulation of the oligomeric state of Pif1 on DNA might determine whether the enzyme functions to unwind double-stranded nucleic acids (helicase) or simply moves along ssDNA (translocase) (24,26–28). To explore these possibilities for Pif1 we have performed experiments to examine whether monomers of Pif1 can translocate on ssDNA, whether a dimer or monomer of Pif1 is sufficient for DNA unwinding activity and whether ssDNA translocation activity can be decoupled from helicase activity and used for other functions.

EXPERIMENTAL PROCEDURES

Reagents and buffers

All chemicals were reagent grade. All solutions were made with distilled and deionized 18 MΩ (Milli-Q) water (Millipore Corp., Bedford, MA). The oligonucleotides were purchased from Integrated DNA Technology (IDT, Coralville, Iowa) and HPLC purified. Homo-oligonucleotides concentrations were determined in 10 mM Tris-HCl pH 8.3, 0.1 mM EDTA using an extinction coefficient of $\varepsilon_{260\ nm} = 8100\ M^{-1}cm^{-1}$ for dT and $\varepsilon_{260\ nm} = 5000\ M^{-1}cm^{-1}$ for Cy3 (27,29). The lengths and location of Cy3 are indicated in the text. For oligonucleotides with mixed sequence composition the extinction coefficient was calculated with the nearest-neighbor method (30). The sequences of the substrates with mixed sequence composition used in this study are shown in Supplementary Table S1. The dsDNA substrates were prepared by annealing a 1:1 ratio of unlabeled to labeled strand in 10 mM Tris-HCl pH 8.3, 100 mM NaCl and 5 mM $MgCl_2$ for 5 min at 97°C and then slow cooling to room temperature.

The concentration of ATP, ADP and ATPgS (Sigma-Aldrich, St. Louis, MO) was determined using $\varepsilon_{259\ nm} = 15\ 400\ M^{-1}cm^{-1}$ (23).

Unless otherwise stated all the experiments were performed at 22°C in Buffer T (50 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$, 0.5 mM DTT and 20% v/v glycerol) with the concentration of NaCl (mM) indicated in the subscript.

Purification of Pif1

Pif1 was purified as previously reported (23). For most of the studies the preparation was stopped after the ssDNA cellulose chromatography step. The addition of the extra step of ATP-agarose affinity chromatography does not change the observed translocation behavior (Supplementary Material). Before use, the protein was extensively dialysed in the indicated buffer and the concentration of Pif1 was determined spectrophotometrically with a Cary 100 (Varian Inc., Palo Alto, CA) using an extinction coefficient $\varepsilon_{280\ nm} = 55\ 000\ M^{-1}cm^{-1}$ (23).

Stopped-flow kinetics

All the stopped-flow experiments were carried out with either a SX.14 or SX.20 Applied Photophysics apparatus maintained at the indicated temperature with a circulating thermostatic bath. Fluorescence intensity measurements monitoring Cy3 changes were performed by excitation at 520 nm and collecting the emission with a 550 nm cut-off filter (Chroma Technology, USA). All the solutions were incubated 7–10 min in the syringes before collection. The reported traces are an average of 4–8 shots collected in pressure-hold mode.

ssDNA translocation

In order to study Pif1 translocation on ssDNA we took advantage of the change in Cy3 fluorescence upon Pif1 binding and we employed an assay initially used to monitor ssDNA translocation of PcrA helicase (31), further developed by the Lohman group to study a variety of ssDNA translocases (27,32–36) and applied to other systems (37–41). Briefly, the assay is based on the empirical observation that when in close proximity of a fluorophore a protein can induce changes in its fluorescence properties (either quenching or enhancement). Therefore, if a protein moves directionally along a lattice (i.e. ssDNA) toward a fluorophore placed at its end, arrival of the protein can elicit a change in the fluorescence of the fluorophore, followed by an additional change in the opposite direction upon dissociation of the protein from the lattice (see (32,42–44) for extensive discussion). The change in the fluorescence of the fluorophore induced by the arrival of the enzyme at the end is then determined for different lengths of the lattice. The longer the lattice the longer the time required for the enzyme to reach the fluorophore, resulting in changes in the observed peak position and shape of the observed time course (32,42–44). Moreover, for an enzyme with low processivity for longer ssDNA substrates a smaller fraction of the protein will reach the end due to the increase probability of dissociating during the cycle of translocation, resulting in a decrease of the amplitude of the observed fluorescence signal.

Unless otherwise indicated all the translocation experiments were done by pre-forming a Cy3-labeled DNA–Pif1 complex at a 2:1 molar ratio of DNA to protein (400 nM DNA and 200 nM Pif1). Under these conditions no more than a monomer of Pif1 is bound to each DNA (Supplementary Figure S1).

DNA unwinding

The top strand (Supplementary Table S1) of the duplex substrates used for these experiments was 5′ end labeled with ^{32}P using PolyNucleotide Kinase under conditions [ATP-g-^{32}P]<<[5′ end] and the concentration determined spectrophotometrically after purification with a BioSpin P-6 column (BioRad, USA). The duplex substrates (D_1 or D_2) were generated with bottom strand without the Cy3 modification (Supplementary Table S1) as described above. Unwinding activity was determined at 22°C in Buffer T_{100} by pre-incubating 400 nM labeled substrates

with a 2.5:1 or 1:2 ratio of Pif1 to DNA and initiating the reaction by equal mixing with 1 mM ATP in the absence or presence of 0.1 mg/ml heparin. After 30 s aliquots were withdrawn and quenched with 250 mM EDTA and a 10-fold excess of unlabeled top strand (trap). After digestions with Proteinase K, the reaction products were resolved on a 12% non-denaturing TBE polyacrylamide gel and imaged with a Storm Phosphorimager (Molecular Dynamics, USA).

Streptavidin displacement

Reactions were performed in Buffer T_{100} with a protocol similar to the one used for other helicases (45 48). Briefly, 400 nM biotinylated and Cy3-labeled DNA substrate is bound to a 4-fold excess of streptavidin and incubated for 3 min. Pif1 is then added at a 2:1 or 1:2 ratio relative to the DNA and allowed to equilibrate for an additional 10 min. Excess free biotin (at a final concentration10-fold higher than streptavidin) or free biotin and ATP (2 mM final concentration) are added and the reaction allowed to continue for 10 min before quenching with an equal volume of 250 mM EDTA, 20% v/v glycerol and 280 mM nts of poly(dT) (to trap Pif1). The free DNA and streptavidin-bound DNA are then separated on a 12% TBE native gel and imaged with a Storm Phosphorimager in fluorescence mode.

RESULTS

A monomer of Pif1 shows evidence for 5′ to 3′ ssDNA translocation

We recently showed that binding of Pif1 to ssDNA induces dimerization of the protein (23). However, in the presence of an excess of ssDNA, a Pif1 monomer is bound to the ssDNA lattice (23). We confirmed this using sedimentation velocity analytical ultracentrifugation of Pif1 DNA complexes formed in the presence of excess nucleic acid, monitoring the sedimentation coefficient of Cy3-labeled ssDNA (Supplementary Figure S1). Analysis of the stoichiometry and sedimentation coefficients shows that no more than one Pif1 monomer is bound to each ssDNA. Therefore, under the conditions employed here any observed ssDNA translocation signal originates from a monomer of Pif1 (see below as well).

Stopped-flow studies of the binding of a monomer of Pif1 to Cy3-labeled ssDNA show a Cy3 fluorescence enhancement. The time course of Cy3 fluorescence after mixing 200 nM Pif1 with 400 nM dT_{20}-Cy3 (Cy3 is at the 3′ end) in Buffer T_{200} is shown in Figure 1a along with the trace after mixing dT_{20}-Cy3 with buffer only. The data show that binding of Pif1 to the ssDNA is accompanied by a Cy3 fluorescence increase without significant loss of amplitude in the dead time of mixing. Moreover, control experiments mixing different combinations of Pif1, DNA, ATP and heparin (Figure 1a, inset) show that 0.1 mg/ml heparin efficiently prevents Pif1 binding to DNA or any ATP-dependent change in Cy3 fluorescence. Therefore, heparin can be used to trap free Pif1 and ensure single-turnover conditions in translocation experiments (27,32).

The time course of Cy3 fluorescence after mixing a pre-formed complex of Pif1 and dT_{20}-Cy3 with 0.1 mg/ml heparin in Buffer T_{200} is shown in Figure 1b (black trace). If Pif1 dissociates from DNA in the presence of heparin it does not rebind to the DNA, therefore the observed Cy3 fluorescence decrease monitors the complete and irreversible dissociation of Pif1 from DNA. The slow apparent rate of dissociation (0.2 0.3 min^{-1}) indicates that Pif1 forms a stable complex with ssDNA in the absence of ATP. The time course is very different when the pre-formed complex is mixed with 1 mM ATP (Figure 1b, red trace). In this case the Cy3 fluorescence increases within the first second, reaches a plateau and then slowly decreases. The initial rise in fluorescence is expected if Pif1 is able to hydrolyse ATP and translocate in a 5′ to 3′ direction toward the fluorophore. Following the initial rise, the signal will reach a plateau if Pif1 undergoes multiple rounds of translocation and rebinding or if it is stably bound to the end of the DNA. The slow decrease in the Cy3 signal corresponds to dissociation of Pif1 from ssDNA once all the ATP is consumed (32,42 44). When the pre-formed complex is mixed with 0.1 mg/ml heparin and 1 mM ATP the Cy3 fluorescence shows yet a different time course (Figure 1b, blue trace). The fluorescence of Cy3 peaks at ~200 ms and then decays to the same level observed for heparin only (fully dissociated Pif1 DNA complex). Under these single-turnover conditions, imposed by the presence of the protein trap, the Cy3 signal change is consistent with the expected behavior of an enzyme that translocates directionally toward the fluorophore (5′ to 3′ in this case) (32,42 44). Indeed control experiments where Cy3 is placed at the 5′ end of the DNA (Figure 1c) show that upon addition of ATP and heparin, the Cy3 fluorescence decreases exponentially (independent of DNA length (data not shown)), consistent with Pif1 moving away from the fluorophore (i.e. with 5′ to 3′ directionality) (32,42 44).

To further test that the Cy3 fluorescence change in presence of ATP and heparin originates from ATP-driven translocation of Pif1 along ssDNA we performed the same experiments in the presence of ATPgS, a non-hydrolysable ATP analog (additional control experiments are shown in Supplementary Figure S2). When a preformed Pif1 dT_{20}-Cy3 complex is mixed with 0.1 mg/ml heparin and 1 mM or 0.1 mM ATPgS in Buffer T_{200} (Figure 1b, dark and light gray), Pif1 rapidly dissociates from DNA. Interestingly, the observed rate of Pif1 dissociation (~3 s^{-1}) in the presence of ATPgS is much faster than for the protein in absence of nucleotides (0.2 0.3 min^{-1}). This is true also for ADP, the product of ATP hydrolysis (Figure 1b, green), although the apparent rate of dissociation is slower than in the presence of ATPgS but still faster than in the absence of nucleotides.

DNA length and ATP concentration dependences show that monomeric Pif1 is a ssDNA translocase

The data in Figure 1 strongly suggest that a monomer of Pif1 can translocate with biased 5′ to 3′ directionally, consistent with the polarity of its unwinding activity (3 5). To