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Determination of the biochemical properties of full-length human PIF1 ATPase

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The PIF1 helicase family performs many cellular functions. To better understand the functions of the human PIF1 helicase, we characterized the biochemical properties of its ATPase. PIF1 is very sensitive to temperature, whereas it is not affected by pH, and the ATPase activity of human PIF1 is dependent on the divalent cations Mg^{2+} and Mn^{2+} but not Ca^{2+} and Zn^{2+}. Inhibition was observed when single-stranded DNA was coated with RPA or SSB. Moreover, the ATPase activity of PIF1 proportionally decreased with decreasing oligonucleotide length due to a decreased binding ability. A minimum of 10 oligonucleotide bases are required for PIF1 binding and the hydrolysis of ATP. The analysis of the biochemical properties of PIF1 together with numerous genetic observations should aid in the understanding of its cellular functions.

Introduction

Helicases play important roles in nearly all aspects of DNA metabolism, acting as critical regulators of genomic stability. Thus, mutations in several DNA helicases of the RecQ family are associated with human diseases, such as Werner, Bloom and Rothmund-Thomson syndromes.[1-3]

The PIF1 subfamily of 5' to 3' DNA helicases[4,5] is conserved from yeast to human.[6] In *Saccharomyces cerevisiae* (*Sc*), *Sc*Pif1 was first isolated due to its involvement in recombination and maintenance of mitochondrial DNA.[7] Pif1 helicase cooperates with base excision repair to protect against spontaneous oxidative mtDNA damage, and *pif1* mutant cells are sensitive to ethidium bromide-induced damage with fragmentation of mtDNA.[8-11] Pif1p possesses both nuclear and mitochondrial targeting signals.[12] The nuclear *Sc*Pif1p performs multiple functions, including the catalytic inhibition of telomerase activity.[13] The overexpression of *Sc*Pif1p results in shorter telomeres, whereas mutation of *Sc*Pif1p results in longer telomeres.[12,14] *Sc*Pif1p also inhibits replication fork progression in rDNA,[15] plays roles in Okazaki fragment procession,[16] and participates in resolving G-quadruplex complexes.[17]

Schizosaccharomyces pombe contains a PIF1 homolog, *pfh1*$^+$,[18] that encodes Pfh1p, which exists as multiple isoforms that localize to either nuclei or mitochondria and is essential for the maintenance of nuclear and mitochondrial DNA.[19] Pfh1p is required for the completion of DNA replication and for the proper response to DNA-damaging agents.[18] Pfh1p also participates in lagging strand DNA maturation and DNA replication through hard-to-replicate sites.[20,21] Due to difficulties in the purification of full-length human PIF1, the first study of hPIF1 was performed on an N-terminally truncated PIF1.[22-24] Subsequently, we reported the first successful purification of full-length human PIF1 protein and documented novel functions of the N-terminal (PINT) domain.[25] Here, we characterized the biochemical properties of the ATPase activity of full-length human PIF1. Biochemical studies of the human PIF1 protein should aid in understanding its physiological function.

Results

Expression of human PIF1 in human tissue. To determine the tissue-specific distribution of *PIF1* mRNA, we performed RT-PCR using commercial mul-titissue cDNA panels (**Fig. 1C**). The expression of *PIF1* appeared low among 16 tested human tissues, demonstrating relatively higher levels in thymus, spleen and testis and the lowest level in prostate. Of the same tested tissues, our results were consistent with previously reported results.[26]

Characterization of ATPase activity. DNA helicases are enzymes that unwind DNA duplexes with the energy released from the hydrolysis of ATP. These enzymes possess ATPase activity, the characterization of which provides important information on their functions as DNA helicases. To determine the biochemical properties of full-length human PIF1, PIF1 containing an N-terminal 6× histidine tag was purified using a previously established procedure (**Fig. 1A**), and the ATPase activity was measured in standard reaction mixtures (Materials and Methods) containing 7.5 μM M13 mp18 ssDNA and 9 nM PIF1 protein. As shown in **Figure 2A**, PIF1 is very sensitive to

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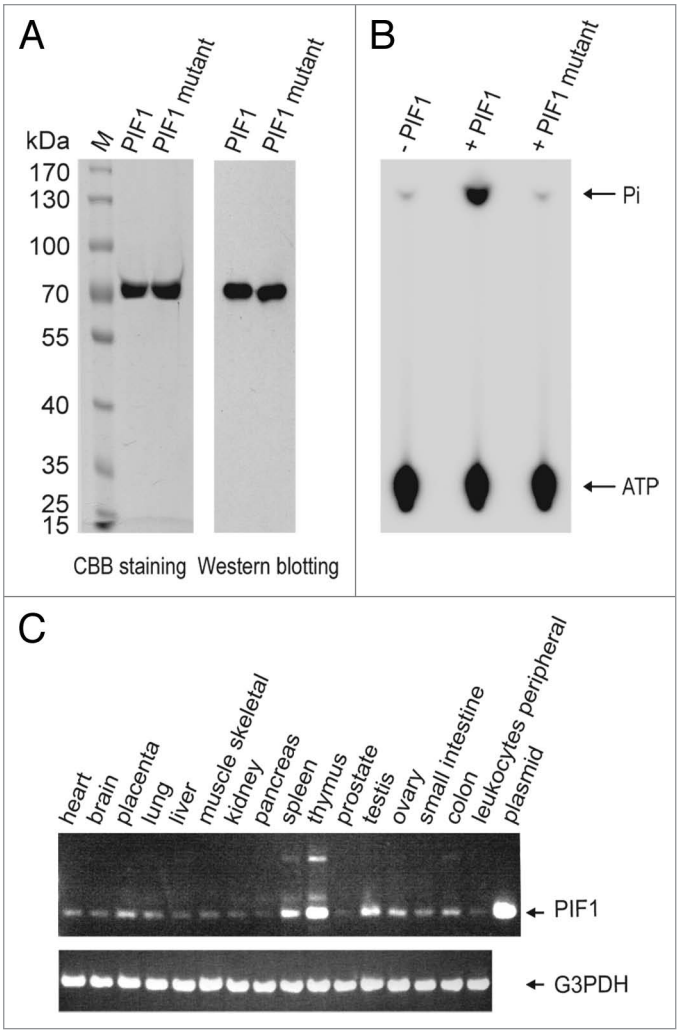

Figure 1. Characterization of PIF1. (**A**) SDS-PAGE of the purified PIF1 and PIF1^{K234A} mutant. Ten micrograms of purified PIF1 and PIF1^{K234A} mutant were loaded on 8% polyacrylamide gel followed by Coomassie brilliant blue R-250 staining and western blotting with an anti-PIF1 antibody. (**B**) ATPase activity of human PIF1 protein and PIF1^{K234A} mutant. ATPase activities were assayed under standard reaction conditions using M13 mp18 ssDNA (3.8 μM in nucleotides) and without PIF1, with PIF1 (140 nM) or PIF1^{K234A} mutant (140 nM) at 30 °C for 10 min. (**C**) Expression of *PIF1*. The expression of *PIF1* mRNA was analyzed using RT-PCR and cDNAs from various human tissues. The expression of *G3PDH* mRNA was independently assayed as a control.

heat. At 25 °C and 30 °C, a similar level of activity was noted, and the amount of products proportionally increased with time within 60 min, which resulted in a total release of 11 000 pmol of phosphate. However, with incubation at 37 °C, the increase in the amount of product began to slow after 7 min and ceased after 15 min incubation, which resulted in a total release of 3000 pmol of phosphate. Incubation at 42 °C resulted in the complete inactivation of the ATPase. Salt, in general, inhibited the ATPase activity of PIF1. A slight stimulation was apparent in the presence of a low salt concentration (less than 1.6 mM), but inhibition occurred at higher concentrations (more than 6 mM) of KCl (**Fig. 2B**). NaCl inhibited the ATPase activity of PIF1 (**Fig. 2B**). However, PIF1 did not appear to be sensitive to pH, as no significant difference in the ATPase activity over a wide pH range from 6.8 to 8.8 could be detected using the Student *t* test for the comparison with the ATPase activity measured at pH 7.5 (**Fig. 2C**). The ATPase activity was dependent on divalent metals, and Mg^{2+} (1.2 mM) stimulated the ATPase activity to the greatest extent. Mn^{2+} (0.5 mM), and Ca^{2+} (0.2 mM) at their optimal concentrations supported 55% and 4% of the Mg^{2+}-supported ATPase activity, whereas 1.2 mM Mn^{2+} only supported 35% of the Mg^{2+}-supported ATPase activity, and 1.2 mM Ca^{2+} did not support the ATPase activity. However, Zn^{2+} did not affect the ATPase activity of PIF1. Because in vivo, single-stranded DNA is typically coated with single-stranded DNA binding proteins, such as RPA in eukaryotes and SSB in prokaryotes, elucidation of whether PIF1 demonstrates ATPase activity in the presence of RPA or SSB is of interest with respect to its cellular functions. Using the determined optimal assay conditions, both RPA and SSB inhibited the ATPase activity of PIF1 over a wide concentration range from 2.0 nM to 280 nM of RPA and from 1.0 nM to 85 nM of SSB, as shown in **Figure 2E** and **F**. Previously we calculated the turnover rate of ATP hydrolysis to be approximately 1000 min^{-1}, which is equivalent to those for *Sc*Pif1 and the fission yeast Pfh1.[5,20,25] An ATPase-dead mutant, PIF1 K234A, in which the lysine in the ATP-binding domain was mutated to an alanine, was also purified by the same purified procedures (**Fig. 1A**), and its ATPase activity was determined. Under standard reaction conditions, the ATPase activity was below the background level (**Fig. 1B**).

Effect of the length of single-stranded DNA on the ATPase activity. Previously, we demonstrated that single-stranded DNA optimally stimulates the ATPase activity of PIF1.[25] To determine the single-stranded DNA length requirements for the ATPase activity, we compared the ATPase activity stimulated by six different oligonucleotides, which ranged from 60 to 10 nucleotides in length that were composed of two bases (CT) to ensure the absence of secondary structures. The results revealed a positive correlation between the ATPase activity and oligonucleotide length (**Fig. 3A**). Decreasing the oligonucleotide length from 60 to 30 nucleotides decreased the ATPase activity 15% with each 10-nucleotide increment, whereas a 20-mer oligonucleotide only supported 50% of the ATPase activity that was stimulated by a 30-mer oligonucleotide. The stimulation of the ATPase activity was minimal using a 10-mer oligonucleotide.

Because these experiments were performed with nearly saturating concentrations of oligonucleotides, the results do not necessarily reflect the affinities of the respective ssDNAs. We therefore determined a kinetic parameter, K_{eff}, that was calculated from ssDNA-titration experiments (**Fig. 3B**) using a hyperbolic curve-fitting program (**Table 1**). The results revealed that decreasing the length from 60 to 30 nucleotides increased the K_{eff} values 2-fold with each 10-nucleotide increment. The K_{eff} value for a 20-mer oligonucleotide was 4-fold higher than that for a 30-mer oligonucleotide, which is consistent with the sudden decrease in ATPase activity. The K_{eff} value for a

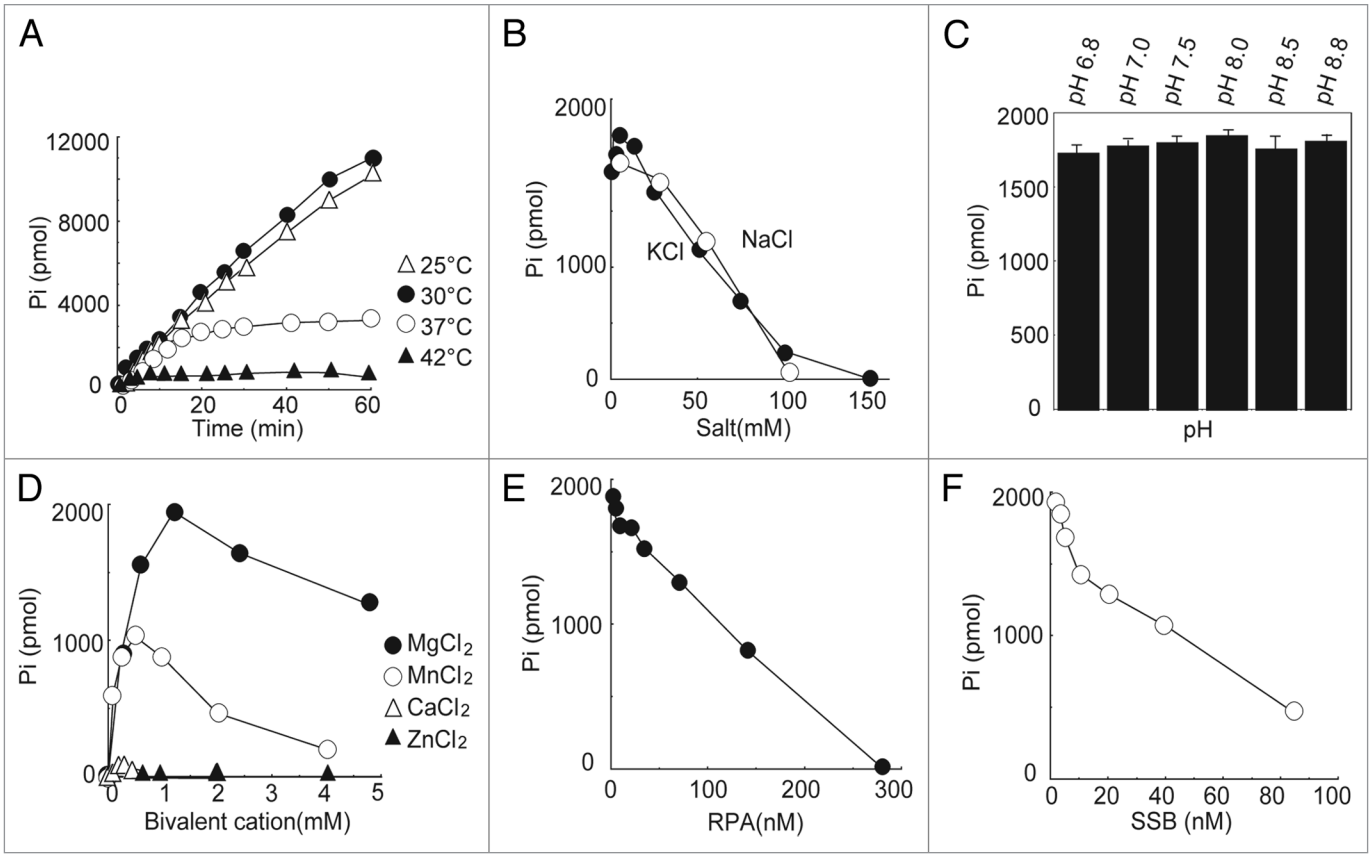

Figure 2. Determination of the biochemical properties of the ATPase activity of human PIF1. (**A**) Effect of temperature on the stimulation of PIF1 ATPase activity. ATPase activities were assayed under standard reaction conditions using M13 mp18 ssDNA (3.8 μM in nucleotides) and PIF1 (9.0 nM) at 25 °C, 30 °C, 37 °C, and 42 °C for the indicated times. (**B**) Effects of salt on the stimulation of PIF1 ATPase activity. Assays were conducted under standard reaction conditions using M13 mp18 ssDNA (3.8 μM in nucleotides), PIF1 (9.0 nM) and increasing concentrations of NaCl or KCl at 30 °C for 10 min. (**C**) Effects of pH on the stimulation of PIF1 ATPase activity. Assays were conducted under standard reaction conditions using M13 mp18 ssDNA (3.8 μM in nucleotides) and PIF1 (9.0 nM) at different pH values and 30 °C for 10 min. The data represent the means ± SD from 3 independent experiments. (**D**) Effects of divalent ions on the stimulation of PIF1 ATPase activity. Assays were measured under standard reaction conditions using M13 mp18 ssDNA (3.8 μM in nucleotides), PIF1 (9.0 nM) and different divalent ions at 30 °C for 10 min. (**E**) Effects of RPA on the stimulation of PIF1 ATPase activity. M13 mp18 ssDNA (3.8 μM in nucleotides) was incubated with an increasing concentration of RPA on ice for 10 min under standard reaction conditions. PIF1 (9.0 nM) was introduced at 30 °C for 10 min. (**F**) Effects of SSB on the stimulation of PIF1 ATPase activity. M13 mp18 ssDNA (3.8 μM in nucleotides) was incubated with an increasing concentration of SSB on ice for 10 min under standard reaction conditions. PIF1 (9.0 nM) was then introduced and incubation continued at 30 °C for 10 min. The data represent 3 independent experiments, and the errors were less than 10%.

10-mer oligonucleotide was found to be 7-fold higher than that for a 20-mer oligonucleotide, which explains its failure to stimulate ATPase activity.

Effects of the length of single-stranded DNA on ssDNA binding ability. We previously established a method to determine the specific DNA binding activity of PIF1 and determined the ssDNA binding activity of PIF1.[25] The decrease in ATPase activity for shorter oligonucleotides might thus be attributable to reduced binding. To measure the ability to bind to different lengths of ssDNA, we performed electrophoretic mobility shift assays (EMSA) using six different oligonucleotides, which ranged from 60 to 10 nucleotides in length that were composed of two bases (CT) to ensure the absence of secondary structures, as in the determination of the optimal ATPase assay conditions. The products were loaded on gels as described in the Materials and Methods section. For the oligonucleotides that contained 60 to 30 nucleotides, the titration of the ssDNA with PIF1 resulted in the formation of PIF1-DNA complexes, which increased with increasing PIF1 concentration (**Fig. 4**). PIF1-DNA complexes were not detected for oligonucleotides containing 20 or 10 nucleotides. The apparent K_d values, which are approximately equal to the protein concentration at which half of the free DNA becomes bound,[27] are listed in **Table 1**. The apparent K_d values for d(CT)60 and d(CT)50 were determined to be approximately 1.29 nM and 2.7 nM, which are in good agreement with their K_{eff} values, 0.05 μM and 0.11 μM (in nucleotides) (**Table 1**), after correction for the oligonucleotide concentration, which results in 0.83 nM and 2.2 nM, respectively. The apparent K_d value for d(CT)40 was estimated to be approximately 15.55 nM, which is slightly higher than the K_{eff} value of 7 nM, which represents the correction of 0.279 μM for the concentration of a 40-mer oligonucleotide. A K_d for d(CT)30 could not be determined due to limited binding.

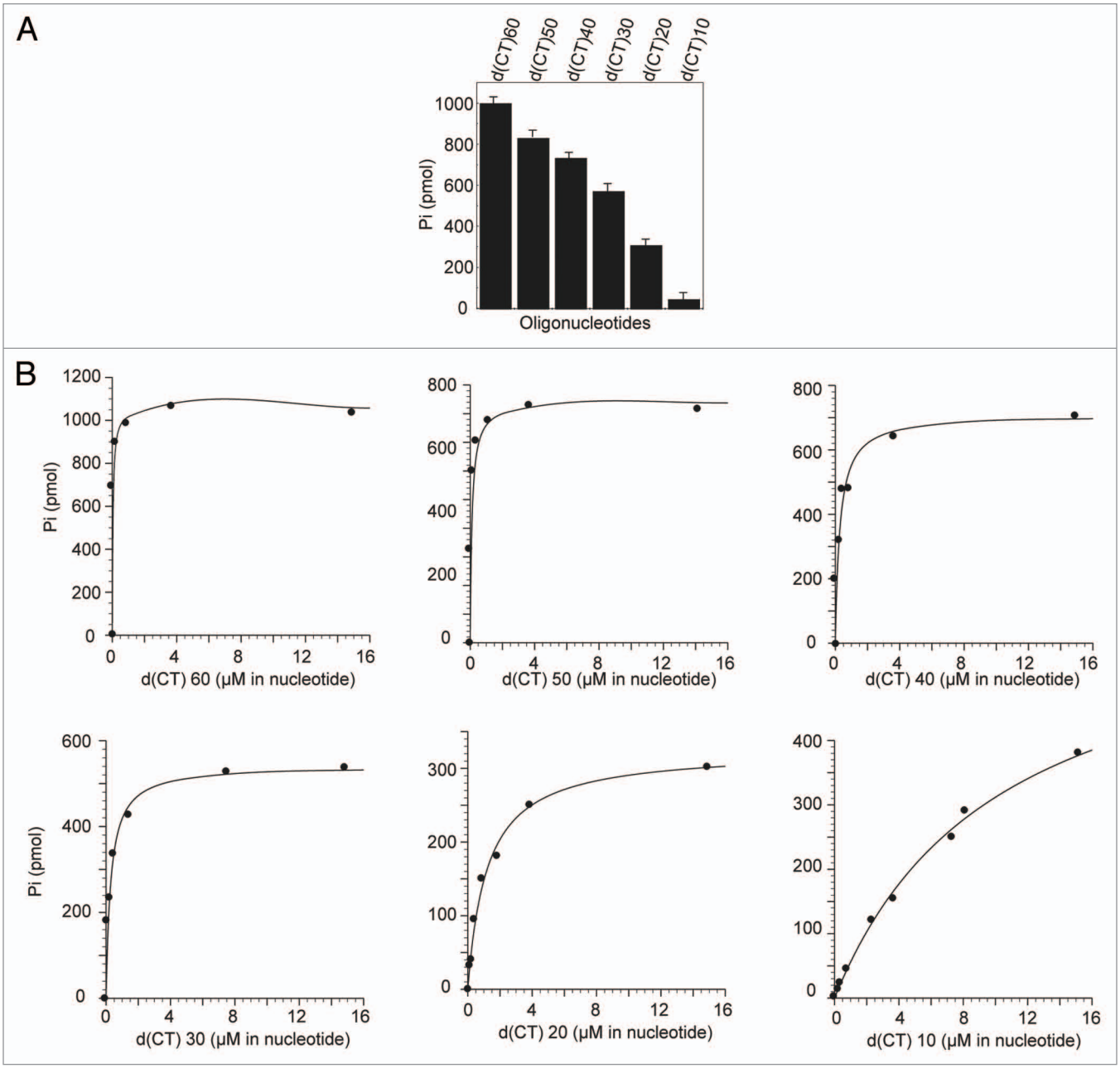

Figure 3. Determination of the effect of different oligonucleotide lengths on PIF1 ATPase activity. (**A**) Effects of different oligonucleotide lengths on PIF1 ATPase activity. ATPase activity was assayed under standard reaction conditions using oligonucleotides (3.8 μM in nucleotides) that ranged in length from 60 to 10 nucleotides and PIF1 (4.5 nM) at 30 °C for 10 min. (**B**) Titration of oligonucleotides for the stimulation of PIF1 ATPase activity. ATPase activity was measured under the standard reaction conditions at 30 °C for 10 min. The protein concentration was 4.5 nM. The data represent 3 independent experiments, and the errors were less than 10%.

Discussion

The present study of the ATPase activity of the human PIF1 helicase revealed a clear correlation with ssDNA binding ability. The ATPase activity of helicases is necessary to provide the energy to enable the translocation of these enzymes along ssDNA and unwind DNA duplexes. Characterization of the ATPase activity provides overall information on the helicase functions of these enzymes. As shown in **Figure 3**, human PIF1 is very sensitive to temperature, which may partially account for the difficulty encountered in its purification. In contrast, the protein is not sensitive to pH, demonstrating a very broad optimal pH. KCl and NaCl, in general, inhibit the ATPase activity of PIF1, which is consistent with previous reports of the fission yeast homolog, Pfh1, and the N-terminally truncated PIF1,[18,19] whereas divalent cations exert a stimulatory effect. In the present study, Mg^{2+} was found to provide the greatest stimulation followed by Mn^{2+}, whereas Ca^{2+} and Zn^{2+} demonstrated only weak effects. In vivo,

single-stranded DNA is typically coated with RPA in eukaryotes and SSB in prokaryotes,[25,28] and both RPA and SSB can inhibit the ATPase activity of PIF1, which suggest that the functions of PIF1 might be regulated by upstream proteins in vivo.

We previously reported that ssDNA binds to PIF1 and greatly stimulates its ATPase activity.[25] In the present study, we demonstrated that the ATPase activity decreases proportionally with a decreasing length of ssDNA. The decreased activity could clearly result from the reduced binding of PIF1 to shorter nucleotides. The apparent K_d values for d(CT)60 and d(CT)50 of 1.29 nM and 2.7 nM, respectively, were in good agreement with the K_{eff} values of 0.83 nM and 2.2 nM, respectively, that are expressed in terms of the oligonucleotide concentration. We suggest that the difficulty in binding shorter oligonucleotides directly leads to a decreased PIF1ATPase activity. Because PIF1 does not sufficiently bind short oligonucleotides that are 10 nucleotides in length, the oligonucleotide d(CT)10 did not appreciably stimulate the ATPase activity of PIF1. Notably, the apparent K_d value for d(CT)30 was too high to be determined, and d(CT)20-PIF1 complexes could not be detected, although d(CT)30 and d(CT)20 were able to stimulate PIF1 ATPase activity to an appreciable extent. This discrepancy might be explained by the insufficient sensitivity of EMSA to detect short oligonucleotide binding to PIF1. It should also be noted that the apparent K_d value for d(CT)40 of 15.55 nM is slightly higher than its K_{eff} value of 7 nM for the ATPase activity.

The PIF1 helicase subfamily appears to perform many cellular functions, ranging from nuclear DNA replication,[15] telomere length regulation,[12-14,26,29,30] mitochondrial genome integrity,[7-11,20] DNA repair,[8-10] Okazaki fragment processing,[16] assisting the replication fork progress through nonnucleosomal protein-DNA complexes.[15] Pif1-like helicases are prone to aggregation and are poorly soluble, which hampers their purification, and in vitro studies have, therefore, been very limited. The results of the present analysis of the biochemical properties of PIF1, together with numerous genetic observations, should aid in a better understanding of its cellular functions.

Materials and Methods

Protein purification and antibody production. His-tagged human $PIF1^{K234A}$ point substitution mutation was constructed by QuickChange® Site-Directed Mutagenesis Kit (Stratagene). His-tagged full-length PIF1 and $PIF1^{K234A}$ were purified from overexpression in *E. coli* BL21 (DE3) cells. The detailed purification procedures have been previously described.[25] RPA was purified as previously described[28] and SSB was purchased from Stratagene (Stratagene). Polyclonal antibodies against PIF1 was generated as previously described.[25]

Multi-tissue RT-PCR. Human MTC Panel I (#636742) and Panel II (#636743) were purchased from Clontech (Clontech), and the forward primer, CACAGATTTG AGGCTATGGA C, and reverse primer, TCAGAGGATT GGGTCCATGT T, were used to amplify *PIF1* using RT-PCR. The primers for *G3PDH* were provided by the manufacturer. Cycling parameters for the analysis of *PIF1* were as follows: 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min. A total of 30 cycles of PCR were performed to amplify *PIF1* and *G3PDH*.

Table 1. Kinetic parameter of the ATPase activity, K_{eff}, and apparent K_d of PIF1 binding to various ssDNAs

DNA	K_{eff}[a] (μM)	K_d (nM)
d(CT)60	0.050	1.29
d(CT)50	0.110	2.70
d(CT)40	0.279	15.55
d(CT)30	0.299	nd[b]
d(CT)20	1.223	nd[b]
d(CT)10	8.034	nd[b]

Kinetic assays to determine K_{eff} values were performed for 10 min in 20-μl reaction mixtures using 4.5 nM of PIF1. Concentrations of ssDNA ranged from 0.15 to 15 μM in nucleotide equivalents. K_{eff} values were evaluated from the plot of the initial velocity vs. the nucleotide concentration using a hyperbolic curve-fitting program with correlation coefficients (R^2) greater than 0.97, which are provided in **Figure 3**. Binding assays to determine the K_d values are described in the legend for **Figure 4**. [a]K_{eff} is expressed in nucleotide equivalents. [b]Not detected.

ATPase assay. ATPase activity was measured in 50 mM TRIS-HCl (pH 8.0), 2 mM DTT, 1.2 mM $MgCl_2$, 0.25 mg/ml BSA, 2 mM [γ-^{32}P]ATP, the indicated amount of DNA, and the indicated amount of protein sample diluted with buffer D (50 mM TRIS-HCl, pH 8.0, 1 M NaCl, 2 mM DTT, 10% glycerol, and 0.1 mg/ml BSA). After incubation at 30 °C for 10 min, the reaction was stopped with 4 μl of 20 mM EDTA (pH 8.0). Two μl of aliquot was spotted onto a polyethyleneimine-cellulose plate (Merck) and developed in 0.3 M LiCl/0.9 M formic acid. The products were analyzed as previously described.[25]

DNA binding assays. End-labeled oligonucleotides were labeled with polynucleotide kinase (New England BioLabs) and [γ-^{32}P] ATP. DNA binding assays were performed using a modified method previously described.[31] The reactions were performed under ATPase assay conditions using 25 pM end -labeled oligonucleotides and indicated amount of PIF1 protein. Samples were incubated on ice for 10 min and loaded on pre-run 5% polyacrylamide gels (79:1 acrylamide/bisacrylamide). The reaction products were analyzed as previously described.[25]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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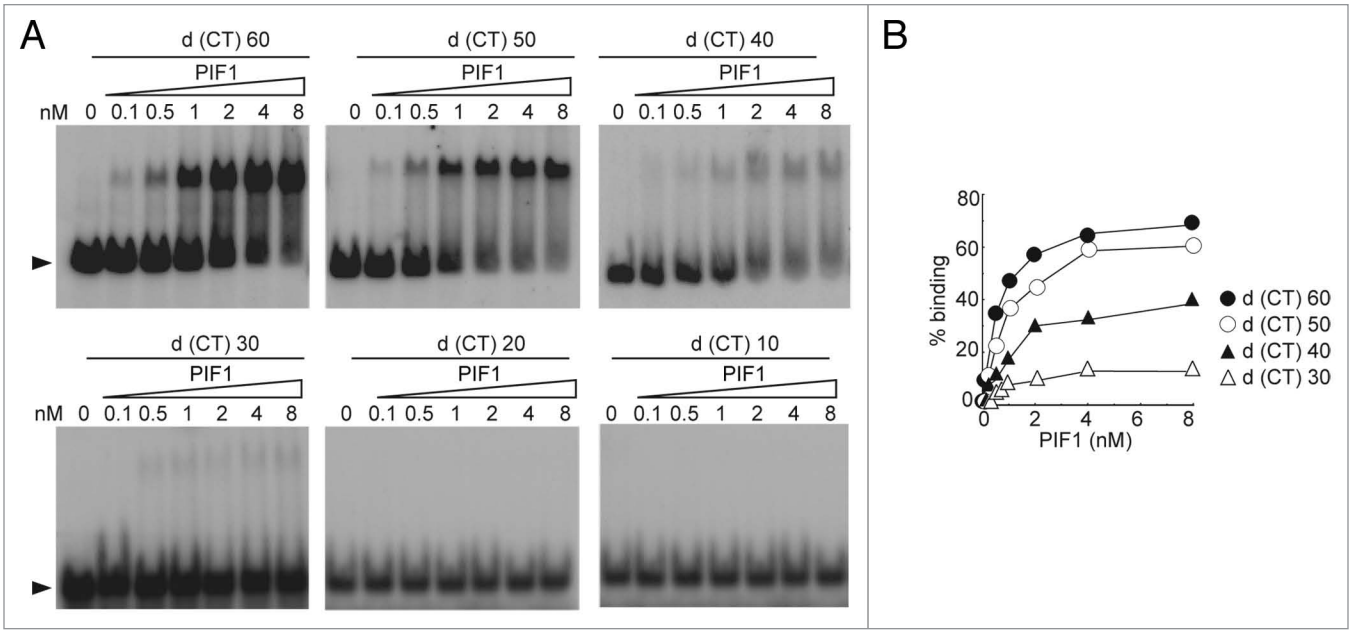

Figure 4. EMSA of PIF1 binding to oligonucleotides of different lengths. (**A**) The binding of PIF1 to oligonucleotides of different lengths. 5'-^{32}P-labeled oligonucleotides of different lengths were incubated with the indicated concentrations of PIF1. Arrowheads show the positions of free DNA. (**B**) Graphic illustration of the quantified data shown for A. The data represent 3 independent experiments, and the errors were less than 10%.

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