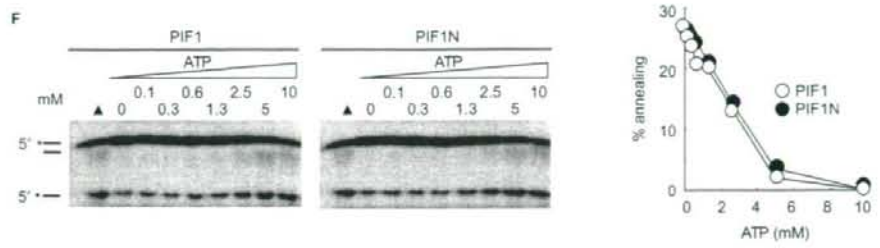

Figure 5. Continued.

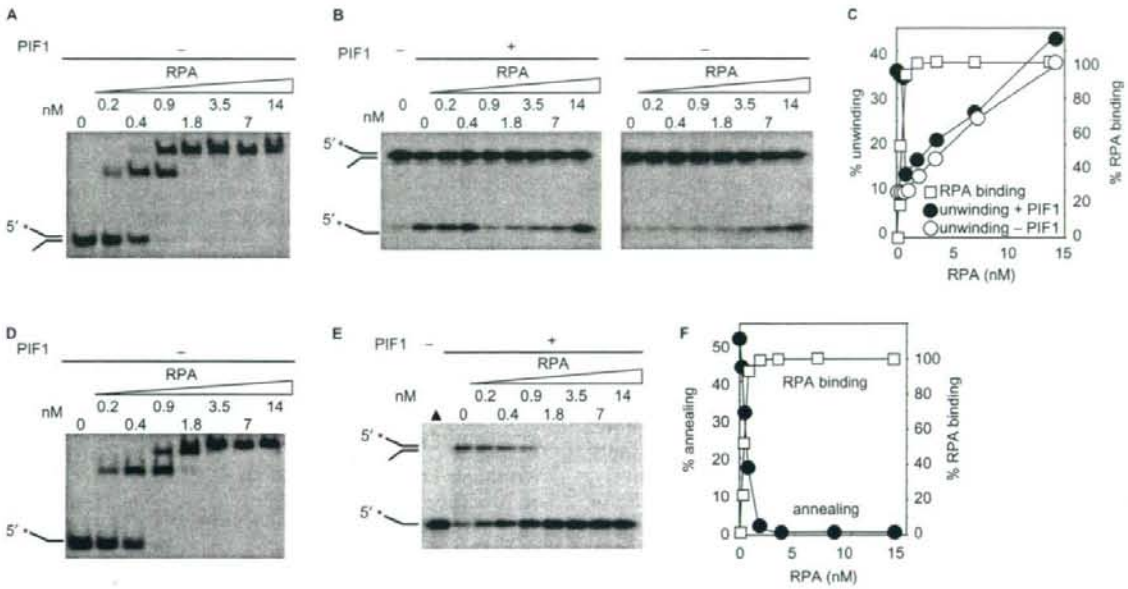

Figure 6. Effects of RPA on the unwinding and annealing reactions. **(A)** EMSA of RPA binding. The fork-structural partial duplex DNA substrate, 3F:4L (0.35 nM) was incubated with RPA at indicated concentrations on ice for 10 min under the standard reaction conditions. **(B)** Unwinding assay in the presence of RPA. The fork-structural partial duplex DNA substrate, 3F:4L (0.35 nM), was incubated with RPA at indicated concentrations on ice for 10 min under standard reaction conditions. Then, PIF1 (16 nM) (left panel) and buffer (right panel) were introduced and incubated at 30°C for 10 min. Reaction products were separated on a 15–25% polyacrylamide gel. **(C)** The quantified data of unwinding reactions in (B) are shown graphically with the RPA binding curve in (A). **(D)** EMSA of RPA binding. The fork-structural partial duplex DNA substrate, 3F:4L (0.35 nM), was boiled for 5 min, then incubated with RPA at indicated concentrations on ice for 10 min under the standard reaction conditions omitting ATP. **(E)** Annealing assay in the presence of RPA. The fork-structural partial duplex DNA substrate, 3F:4L (0.35 nM) (Figure 5A), was boiled for 5 min, then incubated with RPA at indicated concentrations on ice for 10 min under standard reaction conditions omitting ATP. Then PIF1 (30 nM) was introduced and incubated at 30°C for 10 min. Reaction products were separated on a 15–25% polyacrylamide gel. **(F)** The quantified data of annealing reactions in (E) are shown graphically with the RPA binding curve in (D). The errors in the experiments were <10%.

Biochemical analysis of yeast PIF1 homologs has demonstrated that they preferentially unwind forked substrates (1,5,7). We showed that the property is conserved in human PIF1. However, we found that forked substrates were not optimal with respect to stimulation of ATPase activity. Rather nonstructural ssDNA greatly stimulated ATPase activity. The finding that the K_{eff} value for nonstructural ssDNA was lower than for other DNA molecules, including forked structures, suggested preferential binding of PIF1 to ssDNA. From these results, we suggest that PIF1 needs an ssDNA region for loading and a forked structure for entrance to the double strand region by translocation.

We present several lines of evidence that the enzymatic characters of PIF1C reflect the intrinsic properties of the helicase domain. First, PIF1C expressed ATPase activity to the level equivalent to full-length PIF1 (about 1000 min^{-1}) and also equivalent to that for yeast homologs (5,7). Second, full-length PIF1 and PIF1C both showed a similar preference for poly(purine–pyrimidine) and polypyrimidine, but not polypurine, for stimulation of ATPase. This property is also conserved in yeast homologs (5,7). Third, the K_m values for ATP of full-length PIF1 and PIF1C were essentially identical. This is also in agreement with a previous report for N-terminal truncated PIF1, purified as a C-terminus GST-fusion protein (4).

These results suggest that the determined properties of PIF1C are intrinsic to the helicase domain, and could also exclude the possibility that the his-tag at the N-terminal of PIF1C interferes with activities of the helicase domain.

Interestingly, we noted a significant difference between PIF1 and PIF1C with regard to the required concentration of ssDNA for stimulation of ATPase activity. PIF1C needed a 20 times higher concentration and also exhibited much lower unwinding activity. The results suggest that the defects could be attributed to missing functions of the PINT domain. The difference in the K_{eff} values could be due to lower binding affinity of the helicase domain for ssDNA. Consequently, we demonstrated ssDNA binding of PIF1 directly by EMSA. The apparent K_d value, 3 nM was in good agreement with the K_{eff} value of 2 nM when expressed with reference to the oligonucleotide concentration, suggesting that this assay well reflected the functional interaction between ssDNA and PIF1. In this assay, as expected, we demonstrated lower affinity of PIF1C to ssDNA. We suggested that the defect in PIF1C could be due, at least in part, to lower binding affinity for ssDNA, and the PINT domain plays a role for increasing this affinity of the helicase domain. Notably, the K_d value (10 nM) of PIF1C determined by EMSA was still 6 times lower than the K_{eff} value (60 nM in oligonucleotides) for the ATPase assay. We consider the following possible explanations for the discrepancy. We found that PIF1C exhibited a much higher affinity for ssDNA without binding of ATP. With the EMSA, an ATP-free fraction of PIF1C could exist, even in the presence of ATP. Therefore, the results could be an overestimation, due to high affinity binding of the ATP-free fraction of PIF1C. Alternatively, the PINT domain could possess another function for enhancing activity of the helicase domain by modulating the mode of interaction with ssDNA. The higher affinity of PIF1C for ssDNA without binding of ATP could be intrinsic to the helicase domain. The results suggested that ATP modulated the binding mode with ssDNA. The higher affinity to ssDNA before binding of ATP is reduced by binding of ATP. The alteration must be alternatively repeated during turnover of reactions of ATPase. With the full-length PIF1, such alteration due to ATP binding was not detected, suggesting that the PINT domain somehow could suppress the alteration during turnover of ATPase reactions. In this study, we demonstrated that the PINT domain also possesses ssDNA binding activity. We suggest that its enhancement of the activities of the helicase domain is due, at least in part, to this ssDNA binding activity.

We unexpectedly found the PINT domain to further possess ssDNA annealing activity. Among the proteins handled in this study having ssDNA binding activity, including PIF1C, PIF1N and RPA, we could detect annealing activity only with PIF1N. We consider that the annealing activity could be mediated by ssDNA binding, although not attributable to the general effect of high affinity ssDNA binding. Annealing activity has been reported to be associated with the RecQ family helicase in general (39–45). While the RecQ family is distinct from PIF1 family, annealing activity shares similar properties in common. It is located outside of the conserved helicase domains (39,40), is inhibited by RPA, and is ATP-independent or rather inhibited by ATP (39–42,44). We demonstrated that inhibition by ATP is not a consequence of the unwinding reaction, suggesting that it is an intrinsic property of the PINT domain.

At the present time, the biological significance of our findings cannot be readily assessed. Importantly, we showed that RPA inhibited unwinding and annealing reactions, suggesting that these functions of PIF1 might be restricted under particular situations in DNA metabolism. There is a marked difference from RecQ helicases, whose unwinding activity is proficient on RPA-coated ssDNA and stimulated by RPA, although annealing activity is suppressed (39–41,46–48). Notably, unwinding activity of *Sc*Pif1 was stimulated by RPA (1), but that of the fission yeast homolog, Pfh1, was inhibited (7) like human PIF1, suggesting that the outcomes in cells would differ. This could be related to the fact that budding yeast has another member of the PIF1 superfamily, Rrm3, but human and fission yeast have only one. Further analysis of the precise cellular roles of PIF1 should shed light on functions in maintenance of genetic stability.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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