Nucleic Acids Research, 2006, Vol. 34, No. 5 ***1393–1404***
doi:10.1093/nar/gkl029

The human Pif1 helicase, a potential *Escherichia coli* RecD homologue, inhibits telomerase activity

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Received November 24, 2005; Revised February 5, 2006; Accepted February 17, 2006

ABSTRACT

Telomeres, the protein–DNA complexes at the ends of eukaryotic chromosomes, are essential for chromosome stability, and their maintenance is achieved by the specialized reverse transcriptase activity of telomerase or the homologous recombination pathway in most eukaryotes. Here, we identified a human helicase, hPif1 that inhibits telomerase activity. The primary sequence and biochemical analysis suggest that hPif1 is a potential homologue of *Escherichia coli* RecD, an ATP-dependent 5′ to 3′ DNA helicase. Ectopic expression of wild-type, but not the ATPase/helicase-deficient hPif1, causes telomere shortening in HT1080 cells. hPif1 reduces telomerase processivity and unwinds DNA/RNA duplex *in vitro*. hPif1 preferentially binds telomeric DNA *in vitro* and *in vivo*. We propose that the mechanism of hPif1's inhibition on telomerase involves unwinding of the DNA/RNA duplex formed by telomerase RNA and telomeric DNA, and RecD homologues in eukaryotes may have evolved gaining additional functions.

INTRODUCTION

Telomeres are the nucleoprotein structures that are positioned at eukaryotic chromosomal ends. The primary role of telomeres is to insulate the chromosomal ends from both fusion with other ends and from nucleolytic digestion (1,2). The telomeric DNA, which typically consists of tandem repeats of guanine-rich sequences, is essential for telomere function. In humans, each chromosome end bears 5–15 kb of TTAGGG/CCCTAA repeated DNA, which is maintained by telomerase (3) and/or alternative lengthening of telomere (ALT) pathway (2,4).

Telomerase, as found in most organisms, is a specialized reverse transcriptase that uses a small segment of an integral RNA subunit as template to extend the 3′ end of the G-rich strand of telomeres (3,5). In humans, telomerase activity attributed to hTERT (the catalytic protein subunit) and hTR (the RNA subunit) is readily detected in germline cells, high-proliferative cells and many types of cancer cells (6). However, telomerase activity is minimal in most somatic cell lineages (7). Consistent with this, telomeres in most types of human somatic cells shorten with increasing age or with repeated passaging in culture (8,9). The introduction of hTERT into some normal primary human cells has been shown to halt telomere shortening and prevent the cells from entering into senescence (10,11). Although telomerase activation does not induce transformation (12), immortalization of primary human cells and the extensive proliferation of most adult human cancer cells require telomerase activity (13,14). Inhibition of telomerase activity in tumor cells caused telomere shortening and resulted in cell crisis (14–16). For these reasons, repression of telomerase activity might act as an adjuvant therapy for the treatment of human cancer (17).

In most human tumor cells, telomere length is stably maintained by balancing the result of telomere attrition and telomere elongation by telomerase (18,19). The telomere elongation activity of telomerase is determined by both the level of telomerase expression and the accessibility of telomerase to telomere ends (20). Telomere binding protein complex is a major player in the latter factor (20,21). It has been suggested that the human duplex telomeric DNA-binding protein TRF1 and TRF2 recruit TIN2 and hRAP1 to telomeres and nucleate the formation of a specialized chromatin structure to prevent the access of telomerase (22,23). In addition, long telomeres in human cells can form a T-loop structure where the 3′ single-stranded telomere overhang is inserted into the double-stranded telomere region. This renders DNA terminus

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inaccessible to telomerase (24). The formation of T-loop structure appears to be facilitated by TRF1 and TRF2 (24–26). Furthermore, telomeric single-stranded DNA-binding protein hPot1 may also act as a repressor of telomerase (27) since the 3′ end cannot simultaneously binds the hPot1 protein and the telomerase RNA component.

Recently, we determined that the *Saccharomyces* Pif1 helicase and its paralogue Rrm3 helicase are involved in telomere DNA replication (28,29). Rrm3p appears to promote the progression of the replication fork through telomeres (29,30). Pif1p, unlike Rrm3p, inhibits telomere lengthening through the telomerase pathway (28). A proposed mechanism for yeast Pif1p's inhibition of telomerase activity is to release Est2p/Tlc1 complex from telomeric DNA (31). *PIF1*-like genes are found in diverse organisms, for example in *Schizosaccharomyces pombe, Candida maltosa, Caenorhabditis elegans, Drosophila melanogaster* and *Homo sapiens* (28,32). The Pif1p homologue in *S.pombe,* called Pfh1p (Pif1 homologue 1), is essential for cell viability (33). Because the fundamental mechanism of telomere replication is highly conserved from yeast to mammals, the elucidation of the function of telomerase and its regulators in yeast has provided insights that are relevant to the function of their homologues in humans. The yeast Pif1p is the prototype of this helicase subfamily, and the potential human homologue was referred as hPif1 (28). Although the partial sequence comparison of the yeast Pif1p, Rrm3p, Pfh1p and its potential human homologue (hPif1) indicate ~32, 32 and 36% primary sequence identity over 438, 395 and 377 amino acid residues, respectively (28), the existence of human Pif1 helicase was unresolved. To address these issues, we have cloned the cDNA encoding human Pif1, overexpressed and purified the recombinant protein, and characterized its enzymatic activity *in vitro*. Our *in vivo* and *in vitro* data suggested that hPif1 regulates telomere elongation by decreasing telomerase processivity, possibly via a mechanism that involves the unwinding of hTR from telomeric DNA by hPif1.

MATERIALS AND METHODS

Oligonucleotides for assays

Oligonucleotides were synthesized and purified by Takara. The tel18 is $(TTAGGG)_3$. The ran18 is GTTGTAAAACGACGGCCA. The ssDNA is GTTGTAAAACGACGGCCAGTGAAT. The ssRNA is GUAAUCAUGGUCAUAGCUGUUUCCUGUGUGAAAUU. The 5′ end 25mer and 3′ end 35mer oligonucleotides were 5′-GTTGTAAAACGACGGCCAGTGAATT-3′ and GTAATCATGGTCATAGCTGTTTCCTGTGTGAAATT (for DNA helicase assay), or 5′-GUUGUAAAACGACGGCCAGUGAAUU-3′ and GUAAUCAUGGUCAUAGCUGUUUCCUGUGUGAAAUU (for RNA helicase assay).

Human Pif1 constructs, cell lines and antibodies

The full-length human Pif1 coding sequence was PCR amplified from HEK 293 cell cDNA library using the forward primer 5′-TTTGAATTCCATATGCTCTCGGGCATAGAGGCGGCGGCAGGGGAA-3′ and reverse primer 5′-GCTCTAGAATTCCATATGTCAGAGGTTTGGGTCCATGTTCTCCTGGTCTGAGGC-3′. The 1.9 kb PCR products were digested with EcoRI and NdeI, and inserted into pUC19. Sequencing results from two independent clones indicated that both clones contained a single open reading frame (ORF) with several nucleotide differences, which may have been due to PCR errors. These sequences were further analyzed by comparison with the human genome and EST databases. The correct fragments of the two sequenced clones were sub-cloned to obtain the hPIF1 gene with the full-length ORF. The $hPif1^{K234A}$ point substitution mutation was generated by site-directed mutagenesis kit (Clontech). The hPif1, $hPif1^{K234A}$, Myc-tagged hPif1 ($hPif1^{myc}$) or green fluorescent protein (GFP)-tagged hPif1 constructs were transiently or stably transfected into HT1080 cell lines. For hPif1 antibody production, Glutathione *S*-transferase (GST)-fusion fragment of hPif1 N-terminal (76–230 amino acid) or C-terminal (438–550 amino acid) was prepared from *Escherichia coli* and injected into rabbits to generate antisera. The anti-hPif1 antibodies were purified with antigen affinity column.

Expression and purification of recombinant human Pif1 protein

^{GST}hPif1ΔN (167–641 amino acid) or its Walker A mutant $^{GST}hPif1^{K234A}$ΔN was sub-cloned into ScaI and SmaI sites of pEG(KT) and overexpressed in a protease-deficient *Saccharomyces cerevisiae* strain (BCY123). Recombinant proteins were purified according to the protocols described previously (30).

DNA-dependent ATPase assay

The standard reaction mixture (10 μl) contained 20 mM Tris–HCl (pH 7.5), 100 μg/ml BSA, 0.5 mM DTT, 10 mM $MgCl_2$, 333 pM [γ-^{32}P]ATP (3000 Ci/mmol) (Amersham Biosciences), 1 mM cold ATP, 100 ng/μl oligonucleotide and 10 nM of the protein to be tested. Reactions were incubated for 30 min at 37°C and were terminated by addition of 10 μl of 50 mM EDTA. An aliquot of 1 μl of reaction mixture was spotted on a polyethyleneimine cellulose plate (J.T. Baker), which was developed in 0.8 M LiCl. The amounts of [^{32}P]orthophosphate released were visualized using a PhosphorImager (Molecular Dynamics).

DNA/RNA or DNA/DNA helicase assay

Each oligonucleotide was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (New England BioLab). A total of 2.5 pmol of both the ^{32}P-end-labeled 25mer and 36mer oligonucleotides were annealed in a 75 μl reaction mixture with 2.5 pmol of single-stranded M13mp7 DNA, whose hairpin region was digested with EcoRI. The annealed substrates were purified with the Chroma Spin-1000 column (BD Bioscience). The 20 μl reactions contained 1 nM DNA substrate and 50 nM recombinant protein in reaction buffer [20 mM HEPES (pH 7.4), 5 mM $MgAc_2$, 5 mM ATP, 100 μg/ml BSA, 5% glycerol, 1 mM DTT], and were incubated at 37°C for 30 min. Reactions were stopped by addition of 5 μl 100 mM EDTA. Products were analyzed by electrophoresis on a 10% polyacrylamide/TBE gel [89 mM Tris borate (pH 8.3), 2 mM EDTA]. After electrophoresis, the gel was dried and quantified by PhosphorImager.

Gel-shift assay

The reaction mixture contained 100 nM ^{32}P-labeled oligonucleotide, 25 mM HEPES–NaOH (pH 7.5), 100 mM NaCl, 1 mM EDTA, 5% glycerol and 50 nM recombinant protein. Different oligonucleotides were added to appropriate concentration as nonspecific competitor where needed. Reactions were incubated for 30 min at 30°C and loaded on to a 6% PAGE gel for electrophoresis at 4°C. After electrophoresis, the gel was dried and quantified by PhosphorImager.

Inducible gene expression system

HT1080 Tet-Off cell line was purchased from BD Clontech. hPIF1^{myc} or hPIF1^{myc} (K234A) was cloned into the tTA-regulated expression vector pTRE2Hyg (BD Clontech). Cells were stably transfected with either pTRE2-hPIF1 or pTRE2-hPIF1^{K234A} which confers hygromycin resistance. For each construct, about 30 G418-resistant clones were expanded. To induce the overexpression of hPif1 or hPif1^{K234A} in the stable transfectants, cells were grown in the absence of doxycycline in DMEM medium supplemented with either 50 mg/ml hygromycin (*rpi*) or 100 mg/ml G418 (*rpi*). To repress the overexpression of hPif1 or hPif1^{K234A}, the cells were grown in the presence of doxycycline (500 ng/ml) in DMEM medium. The hPif1 protein was detected by immunoprecipitation–Western blotting using anti-Myc antibody or anti-hPif1 antibody.

Telomeric repeat amplification protocol assay

Approximately 10^6 HT1080 cells were lysed in 1 ml of CHAPS buffer [10 mM Tris–HCl (pH7.5), 1 mM EDTA, 0.5% CHAPS, 10% glycerol] and $\sim 10^2$ cells were used in each assay. The telomerase activity was assessed using the TRAP-eze telomerase detection kit (Chemicon) according to the manufacturer's instruction. To examine the effects of indicated proteins on telomerase activity *in vitro*, various amounts of proteins were added into the reaction and incubated for 30 min at 30°C before subjecting to PCR amplification. Products were separated on 12.5% native gel, which was then stained with SYBR Green.

In vitro reconstitution of human telomerase

hTERT was expressed from phTERT and hTR subunit from phTR+1 using the TnT quick-coupled transcription /translation system (Promega). Each 100 µl reaction contained 80 µl of TnT-quick mix, 5 µl of PCR enhancer, 2 µl of 1 mM methionine and 2 µg of supercoiled phTERT and BamHI, HindIII-cut phTR+1 plasmid DNA. After incubation at 30°C for 2 h, the product was mixed with 10 µl of 100% glycerol and stored at −80°C.

Direct telomerase activity assay

The 20 µl reaction contained 8 µl of reconstituted telomerase complex, 50 mM Tris-OAc (pH 8.3), 50mM KCl, 1 mM $MgCl_2$, 5 mM DTT, 1 µM 5′-biotinylated $(TTAGGG)_3$ (Takara), 1 µM [α-^{32}P]dGTP (3000 Ci/mmol), 2.5 µM cold dGTP, 1 mM dATP, 1 mM dTTP. The reaction mix was incubated at 30°C for 30 min. The elongation step was terminated by incubating with 50 µl of stopping buffer (10 mM EDTA, 0.1 mg/ml RNase A, 0.5 mg/ml Proteinase K, 0.1% SDS) for 30 min. Then 50 µl of pre-washed Streptavidin-coated Dynabeads M-280 Streptavidin suspension (Dynal Biotech) in buffer A [10 mM Tris–HCl (pH 7.5), 1 M NaCl, 0.5 mM EDTA] was added. A total of 1 fmol of radiolabeled and biotinylated TGTGTGTGGG was also included as a loading control. The elongation product was immobilized onto the magnetic beads at room tempreture for 30 min and followed two times wash with buffer A and three times wash with buffer B [10 mM Tris–HCl (pH 7.5), 0.5 mM EDTA]. The beads were resuspended in 95% deionized formamide and boiled for 20 min. Telomerase reaction products were analyzed by 15% polyacrylamide-urea gel electrophoresis. After electrophoresis, the gel was dried and quantified by PhosphorImager.

For processivity analysis, the radioactive signal for each band detected by PhosphorImager was quantified with ImageQuant software (Molecular Dynamics). The processivity was determined according to the formula: $P_i = \frac{\sum_{j=i}^{n} T_j}{T_i} (i = 1, 2, 3, 4, \ldots, n)$, where P_i means that the sum of radioactive signal for each band above position $+i$ (including position $+i$) is divided by the radioactive signal of position $+i$, where T_i represents the radioactive signal for the primer $+i$ position; the P_i value reflects the relative ability of the enzyme to pass through the position $+i$.

Genomic blotting and telomere length estimation

Genomic DNA was isolated from cells as described (19) at the indicated population doubling (PD), digested to the completion with HinfI/RsaI and quantified by fluorometry using Hoechst 33258 dye. DNA was size-fractionated on a 0.7% agarose gel, transferred to HyBond N+ membranes (Amersham), crosslinked by ultraviolet (UV), and probed with ^{32}P-labeled repeated telomeric probe. The membrane was exposed to a phosphor screen (Molecular Dynamics). The median length of telomeric restriction fragments was determined as described in Li *et al*. (34) using ImageQuant software after scanning with a PhosphorImager.

Immunoprecipitation

Cells were scraped, washed with cold phosphate-buffered saline (PBS), and lysed in RIPA lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1.0% Na-deoxycholate, 1.0% NP-40, 0.1% SDS]. Cell extract from 10^7 cells was incubated with either 5 µg of pre-immune sera or polyclonal anti-hPif1-N (76–230 amino acid) antibodies overnight at 4°C. The antibody–protein complexes were precipitated by Protein A agarose beads (Amersham Biosciences), washed four times with NETN buffer [20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40], and eluted with SDS–PAGE loading buffer. The eluted proteins were separated by SDS–PAGE, transferred to nitrocellulose membranes (Amersham Biosciences), and probed with polyclonal anti-hPif1-C (438–550 amino acid) antibodies.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described (27). Briefly, 10^6 cells were trypsinized and lysed. The DNA and associated proteins in the extracts were crosslinked, fragmented by sonication, and then immunoprecipitated with either

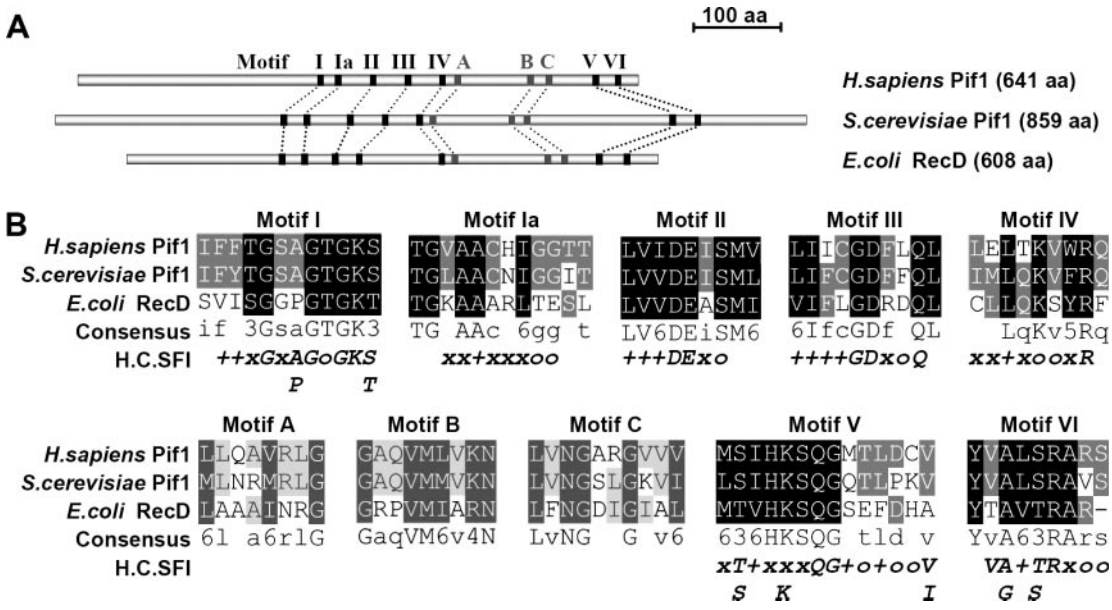

Figure 1. Alignment of amino acid sequences of Pif1 and the *E.coli* RecD protein. (**A**) Alignment of conserved motifs in hPif1, yPif1 and *E.coli* RecD proteins. Roman numerals indicate helicase motifs and the letters indicate unknown motifs. (**B**) Sequence conservation of conserved motifs in hPif1, yPif1 and *E.coli* RecD proteins. H.C.SFI: helicase consensus of superfamily I. +, hydrophobic; o, hydrophilic; 3, Ser/Thr; 4, Lys/Arg; 5, Phe/Tyr/Trp; 6, Leu/Ile/Val/Met; x, any amino acid.

pre-immune sera, anti-TRF1 (*ab1428,* Abcam Inc.) or polyclonal anti-hPif1 antibodies. The DNA was then purified, denatured at 65°C and dot blotted on to a Hybond N^+ membrane (Amersham Bioscience). The membrane was washed with 0.5 M Tris–HCl (pH 8.0) containing 150 mM NaCl, air dried, blocked and hybridized with ^{32}P-labeled telomere probe or Alu probe (5′-GCAGTGAGCCGAGATCGCGC-CACTGCACTCC-3′), and exposed on phosphor screen (Molecular Dynamics). The quantification was done with the ImageQuant software. All lysates were normalized for cell number. For the total telomeric DNA samples, three 12.5 µl aliquots (corresponding to one-eighth of the amount of lysate used in the immunoprecipitations) were processed along with the rest of the samples at the step of reversing the crosslinks. The average of the telomeric signal in three total-fractions were taken for the reference value (total DNA), and the percentage of each immunoprecipitation sample was calculated based on the signal relative to the corresponding total DNA signal.

RESULTS

Molecular cloning of cDNA encoding human Pif1

We previously reported that the putative human Pif1 and yeast Pif1p share 32% identity over a 438 amino acid region (28), as a hPIF1 cDNA clone with the full-length gene was not available then. A *Drosophila* cDNA clone (accession no. NM_134938) which encodes a homologue of yPif1p was later found by a homology search in GeneBank. When the *Drosophila* clone was used to search the NCBI database, we found two human clones (accession nos: AK026345 and BC033254), which have homology with the N and C terminus of yPif1p, respectively. In addition, these two human clones lie in the chromosome 15q22.31, ~9 kb apart, suggesting that they may comprise parts of the hPIF1. Subsequently, a 1.9 kb hPif1 gene was obtained by PCR from a HEK 293 cell cDNA library, and cloned into expression vectors (see Materials and Methods). The deduced amino acid sequences indicated that the hPIF1 is a 641 amino acid protein with a predicted molecular weight of ~71 kDa, and contains seven conserved motifs characteristic of DNA helicases (Figure 1A and Supplementary Figure 1) (35).

hPif1 is a potential homologue of *E.coli* RecD

Helicases utilize the energy of NTP hydrolysis to catalyze the unwinding of double-stranded nucleic acids, and play important roles in many cellular processes (35). Most helicases from various organisms have been classified into superfamily I and II (SFI and SFII), and contain seven conserved helicase motifs (I, Ia, II, III, IV, V and VI) (36). These motifs are usually found clustered in a relatively short region over 300–500 amino acids long. The conserved helicase motifs of I to VI in SFI share little similarity with the corresponding motifs of that in SFII. The high degree of conservation of Pif1 subfamily helicases in *S.cerevisiae, S.pombe, C.maltosa, C.elegans, D.melanogaster*, *Mus musculus and H.sapiens* (28,32) suggests that they might have evolved from a common ancestor which plays fundamental and conserved roles in nucleic acid metabolism.

To seek cousins of Pif1, we searched multiple gene and protein databases. Because the conserved helicase motifs are short and degenerate, it is difficult to identify homologues even in the same superfamily when the full-length sequences of helicase are analyzed with traditional BLAST or FASTA computer programs. Instead, we searched the Cluster of Orthologous Groups of proteins (COG) database, and discovered that hPif1, as well as other members of Pif1 subfamily, clustered with RecD of *E.coli*. As a result of the large phylogenetic distance, the sequence identity between them was low (<20%).

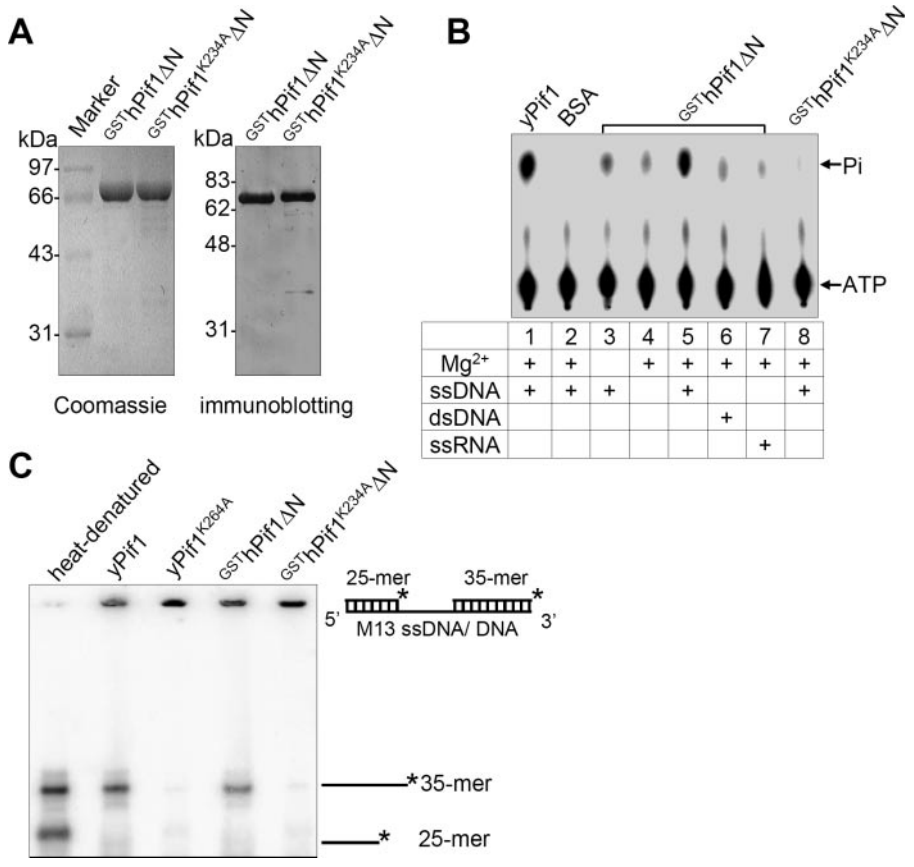

Figure 2. Enzymatical characterization of recombinant hPif1 protein. (**A**) Expression and purification of recombinant ^{GST}hPif1ΔN or ^{GST}hPif1^{K234A}ΔN protein in *S.cerevisiae*. Purified protein were resolved by 10% SDS–PAGE and detected by Coomassie blue staining (left panel) or immunoblotting (right panel). (**B**) ATP hydrolyzing activity of ^{GST}hPif1ΔN or ^{GST}hPif1^{K234A}ΔN protein. A total of 333 pM radiolabeled ATP and 1 mM cold ATP were incubated with 10 nM indicated protein and other reagents as shown. The products were developed in a polyethylimine (PEI) cellulose plate and visualized on a Molecular Dynamics PhosphorImager. (**C**) DNA helicase polarity assay of ^{GST}hPif1ΔN or ^{GST}hPif1^{K234A}ΔN proteins. The DNA substrate was linearized M13mp7 single-stranded DNA to which radiolabeled 36mer and 25mer DNA oligonucleotides had been annealed. The substrate was incubated with 50 nM indicated protein in reaction buffer and examined by 12% native gel. Asterisk represents the radiolabeled site at 5′ end of primers.

However, as the COG database does not apply any constraint of an arbitrarily-chosen statistical cut-off, but rather uses the best-hit rule, which accommodates both slow- and fast-evolving proteins (37). Therefore, we could readily search for putative ancestral proteins of most eukaryotic helicases, and classify the members of superfamilies into different COGs (Supplementary Table 1). Because hPif1 identified RecD in our COG search, we then compared the protein sequences of hPif1 and RecD. The helicase motifs of hPif1 have high sequence identity with the consensus helicase motifs in RecD proteins (Figure 1 and Supplementary Figure 1). In contrast to other members of SFI, Pif1 subfamily and prokaryotic RecD subfamily do share some other conserved motifs, designated as motifs A, B, C (Figure 1 and Supplementary Figure 1), whose functions are unknown, in addition to the helicase motifs. All these data imply that eukaryotic Pif1 subfamily belongs to RecD subfamily.

hPif1 is an ATPase and a 5′ to 3′DNA helicase

In general, homologues share similar biochemical properties. As previously reported, *E.coli* RecD is a subunit of RecBCD complex, and possesses ATP-dependent 5′ to 3′DNA helicase activity (38,39). To characterize hPif1 activity *in vitro*, we tried to overexpress and purify full-length recombinant hPif1 protein. However, the full-length hPif1 was difficult to overexpress and/or purify even when expressed in heterologous expression systems, including those utilizing bacteria, yeast and insect cells. According to the full-length sequence alignment of Pif1 subfamily and RecD (data not shown), the N-terminals of these proteins are not conserved, suggesting that they may not be essential for the conserved enzymatic activity. Thus, we overexpressed the recombinant N-terminal deleted hPif1 that contains the seven conserved helicase motifs with an N-terminal GST fusion (^{GST}hPif1ΔN) in *S.cerevisiae* and purified it to near homogeneity. A mutant ^{GST}hPif1^{K234A}ΔN, in which the conserved Lysine in the ATP binding domain (helicase motif I in Figure 1B) was changed to an Alanine, was overexpressed and purified in parallel (Figure 2A).

Subsequently, we measured ATPase activity of the recombinant proteins, and found out that the recombinant ^{GST}hPif1ΔN could efficiently hydrolyze ATP in the presence of both Mg^{2+} and single-stranded DNA (Figure 2B, lanes 3–5). Little ATPase activity of ^{GST}hPif1ΔN was detected in the presence of RNA or double-stranded DNA (Figure 2B,

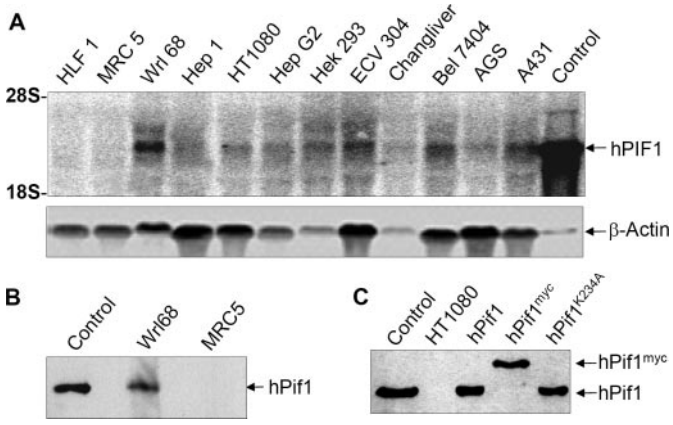

Figure 3. Expression of hPIF1 in transformed human cell lines. (**A**) Northern blot analysis of hPIF1 expression in 12 cell lines, and the RNA from 293T cells transiently transfected with hPIF1 plasmid as positive control. The probe was a fragment of hPif1 gene (1335-1926 nt). Actin was detected as internal control. (**B**) Western blot analysis of the endogenous hPif1 immunoprecipitated from Wrl68 and MRC5 cells, and total extract of transient expression of hPif1 in 293T cells was used as positive control. The primary antibody for Western analysis was against hPif1 C-terminal (438–550 amino acid), while the antibody for immunoprecipitation was against hPif1 N-terminal (76–230 amino acid). (**C**) Western analysis of overexpression of hPif1, $hPif1^{K234A}$ or $hPif1^{myc}$ in HT1080 stable cell lines as in (B).

lanes 5–7), indicating that hPif1 has a preference for single-stranded DNA. No ATPase activity was detected with the $^{GST}hPif1^{K234A}\Delta N$ (Figure 2B, lane 8). Thus, hPif1 confers Mg^{2+}-dependent and single-stranded DNA-stimulated ATP hydrolysis activity.

To analyze hPif1's helicase activity, a standard helicase polarity assay (30) was performed, using a linearized single-stranded M13mp7 DNA annealed with a 35mer and 25mer oligonucleotide at the 5′ and 3′ ends, respectively. DNA helicases will move along the single-stranded DNA and displace at least one of the oligonucleotides. As shown in Figure 2C, recombinant $^{GST}hPif1\Delta N$, as well as yPif1p, displaced the 35mer oligonucleotide, while the ATPase-deficient $^{GST}hPif1^{K234A}\Delta N$ did not. We conclude that the hPif1 is an ATP-dependent 5′ to 3′DNA helicase. Collectively, these data indicate that recombinant hPif1 has enzymatic properties *in vitro* that resemble those of both yPif1p and RecD protein.

Overexpression of ATPase/helicase-functional hPIF1 results in telomere shortening

The messenger RNA of hPif1 has been reported to be present in some human tissues (40,41), however, these reported clones matched only with either N or C terminus sequence of our clone. To analyze the expression of hPif1 mRNA and protein, we examined 12 transformed cell lines derived from different human tissues. The hPif1 transcripts were detected in most of tested cell lines (Figure 3A); however, the protein was undetectable when the total cell lysates were subjected to Western blotting (data not shown). We then enriched the hPif1 by immunoprecipitation, following Western blotting and finally detected a faint band only in the extract of Wrl68 hepatocytes (Figure 3B), which corresponded to the size of hPif1 detected in transiently transfected HT1080 cells. This result suggests that the expression of hPif1 protein in tested cells is very limited. To examine whether hPif1 could be ectopically overexpressed in human cell lines, we transfected hPif1 construct into HT1080 fibrosarcoma cells, in which endogenous expression of hPif1 protein was minimum, to establish stable cell lines. The ectopic expression of hPif1 or $hPif1^{myc}$ was detected as a single band in the extract of the stable cell lines by Western blotting after immunoprecipitation (Figure 3C), suggesting that hPif1 has only one form, unlike yPif1 which exists as both nuclear and mitochondrial forms with different molecular sizes (42). Overexpressed GFP-fused hPif1 was observed in nucleus (data not shown), suggesting that no mitochondrial form of hPif1 exists.

To investigate the role of hPIF1 in telomere DNA replication, we took advantage of Tet-Off HT1080 cell line (19,20), a telomerase-positive human fibrosarcoma-cell line with a stable telomere length, for inducible expression of full-length hPif1 containing a C-terminal Myc epitope ($hPIF1^{myc}$) or its ATPase-deficient mutant $hPIF1^{myc}$(K234A) (Figure 2B). Western analysis with anti-hPif1 antibodies showed that doxycycline controlled the expression of wide-type or mutant hPif1 proteins in clonal Tet-Off HT1080 cell lines transfected with the constructs (Figure 4A and B). At PD one after removal of doxycycline, the expression of hPif1 was induced and remained relatively stable thereafter. The expression of hPif1 was repressed with the re-introduction of doxycycline back into the culture medium. Expression of wild-type or mutant hPIF1 proteins did not affect the overall growth rate of the cells for at least 150 PDs (data not shown).

We then studied the effects of long-term overexpression of $hPIF1^{myc}$ or $hPIF1^{myc}$ (K234A) on telomere length in these transfected cells. Cells overexpressing hPif1 showed gradual telomeric shortening after induction. The change of telomere length was not discernible at one PD when the expression of hPif1 was already fully induced, but a shortening of terminal restriction fragments was obvious between 15 and 35 PDs (Figure 4C and E). The loss of telomeric sequences was also evident from a reduction in the TTAGGG hybridization signal (Figure 4C and E). Telomere shortening was detected in all five tested cell lines that overexpressed $hPIF1^{myc}$ (Table 1). In these cells (T2, T9, T10, T12 and T15), the decline of telomere length was dependent on the absence of doxycycline. After extensive passaging the cell lines (55 PDs), the telomeres shortened by 0.5–1.5 kb, and the rate of telomere shortening varied in the different cell lines from 10 to 27 bp per PD (Table 1). A control Tet-Off HT1080 cell line transfected with mutated hPIF1 had stable telomeres over 130 PDs and there was no effect on telomere length after inducing $hPIF1^{myc}$ (K234A) overexpression (Figure 4D and F). These results indicate that hPIF1 negatively regulates telomere length and that such inhibition requires the ATPase/helicase activity of hPif1.

Repression of hPif1 overexpression recovers telomere length

It has been suggested that the establishment of stable telomere length could be determined by dynamic factors such as telomerase activity, the rate of telomere shortening and the levels of other factors that control telomere length (20). The overexpression of hPif1 for more than 35 PDs seemed to

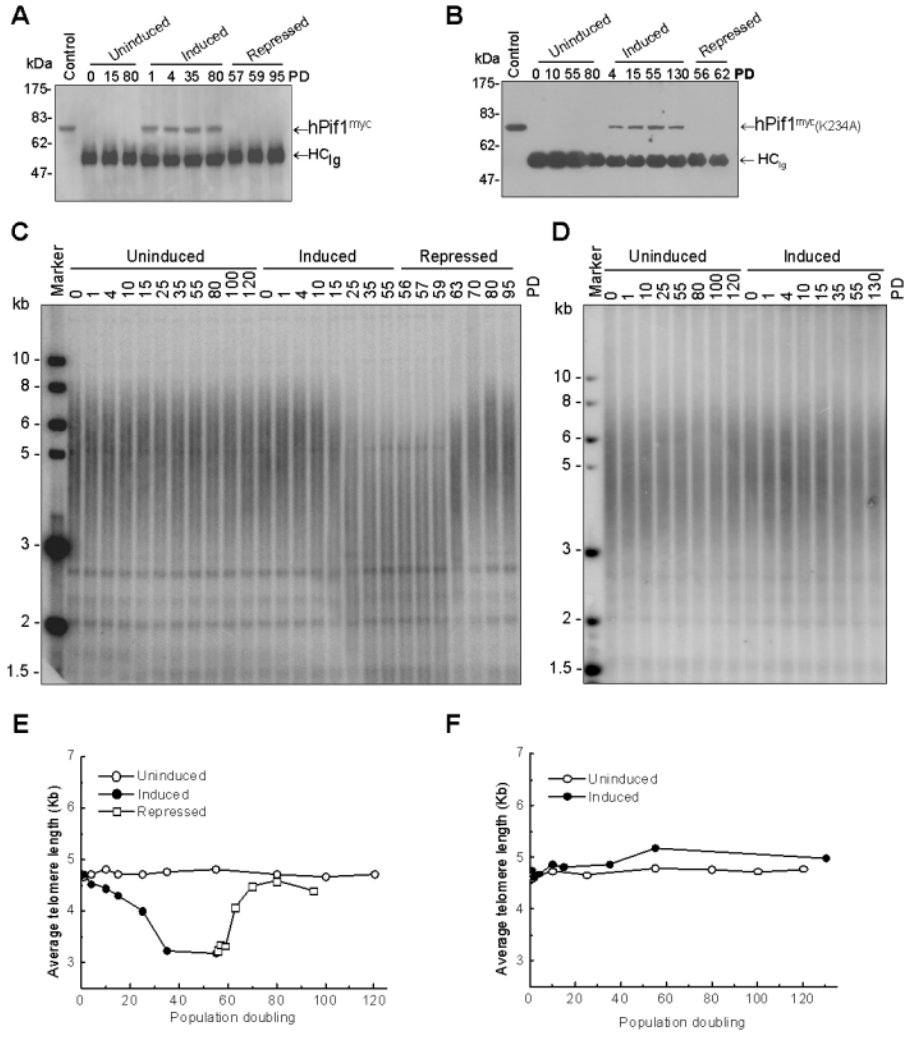

Figure 4. hPif1 inhibits telomere elongation *in vivo*. (**A** and **B**) Tetracycline-regulated expression of hPif1^{myc} (A) and hPif1^{myc} (K234A) (B) proteins in a hPif1^{myc}-containing Tet-Off HT1080 cell line (T15) and a helicase-deficient hPif1^{myc} (K234A)-containing Tet-Off cell line (Tm14), respectively. Immunoblotting analysis of the hPif1^{myc} and hPif1^{myc} (K234A) overexpression in cells was performed under induced, uninduced and repressed conditions. Whole-cell extract at the indicated PDs was immunoprecipitated using anti-hPif1-N (76–230 amino acid) antibody. Immunoprecipitates were detected with anti-hPif1-C (438–550 amino acid) antibody. All cells were grown in parallel with or without doxycycline; repressed cells were with doxycycline after passaging cells for 55 PDs at induced conditions. Total extract of transient expression of hPif1 in 293T cells was used as a positive control. HC_{Ig} (heavy chain of immunoglobulin) served as loading controls. (**C**) A hPif1^{myc}-containing Tet-Off HT1080 cell line (T15) was grown for indicated PD in media with under uninduced or repressed (with doxycycline) or induced (without doxycycline) conditions, and genomic DNA was isolated at the indicated PD. The DNA was digested with HinfI/RsaI, was separated on an agarose gel, Blots were probed with a TTAGGG repeat probe to detect telomeric restriction fragments. Molecular weight standards are indicated to the left of the gels. (**D**) A helicase-deficient hPif1^{myc} (K234A)-containing Tet-Off cell line (Tm14) was grown for 120–130 PD under uninduced or repressed or induced conditions. Genomic DNA samples from the cells at the indicated PD were subjected to TRF analysis as described above. (**E** and **F**) Representative genomic Southern blots of (C) and (D). The plot represents mean telomere length values. Open circles: uninduced conditions; filled circles: induced conditions; open square: repressed conditions (at and after PD55).

Table 1. Telomere length affected by hPif1

Tet-Off HT1080 clones	hPif1 construct	ΔTelomere length at PD 55		Shorten ratio (bp/PD)	Elongate ratio (bp/PD)
		+Dox (kb)	−Dox (kb)		
Tm11	hPif1^{myc}(K234A)	+0.1	+0.3	0	+4
Tm14	hPif1^{myc}(K234A)	+0.2	+0.3	0	+5
T2	hPif1^{myc}	0	−0.5	−10	+13
T9	hPif1^{myc}	+0.1	−0.7	−13	+18
T10	hPif1^{myc}	−0.1	−0.8	−15	+20
T12	hPif1^{myc}	+0.1	−1.1	−20	+28
T15	hPif1^{myc}	+0.2	−1.5	−27	+38

[a]PD: population doubling; Dox: doxycycline.

promote a new equilibrium in telomere length (Figure 4C and E). At this stage, telomere length became stable and no further changes were observed when the culture was grown for an additional 50 PDs (data not shown). If the changes in telomere length were caused by hPif1 overexpression, suppression of hPif1 would release the inhibition. Therefore, doxycycline was added in the culture medium to repress the expression of hPif1, and the telomeres in the HT1080 Tet-Off cells were analyzed. In the T15 clone (Figure 4C and E), shortened telomeres showed gradual elongation over 40 PDs at a rate of 38 bp per PD. The short telomeres in other clones (T2, T9, T10 and T12) also showed similar increase in length, and were eventually stabilized (Table 1), indicating that silencing of ectopic

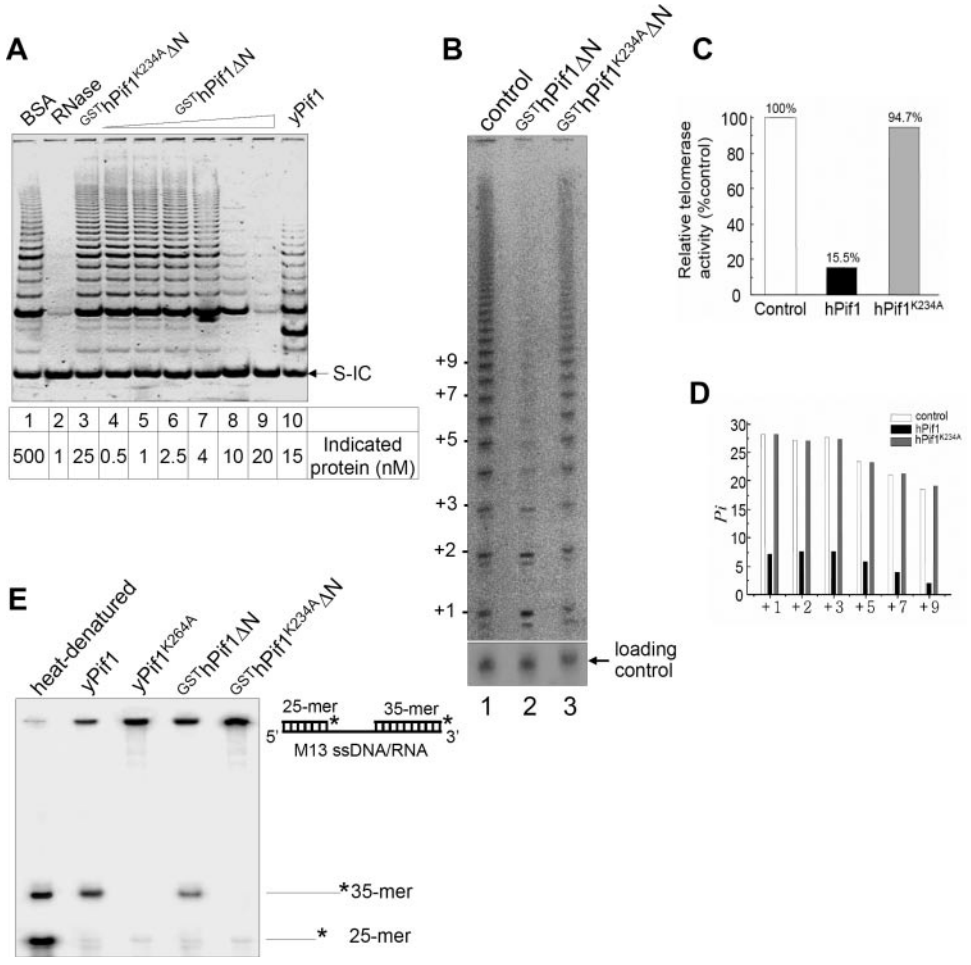

Figure 5. hPif1 inhibits telomerase activity *in vitro*. (**A**) TRAP assay of telomerase. Each reaction contains extract from ~100 HT1080 cells. Protein was added as indicated. The equivalent cell lysate treated with RNase A acted as a negative control. Arrows point to the 36 bp internal control (S-IC) for PCR amplification. (**B**) Direct telomerase activity assay performed with 1 μM $(TTAGGG)_3$ in presence or absence of ^{GST}hPif1ΔN or ^{GST}hPif1^{K234A}ΔN. Lane 1 (control): *in vitro* reconstituted telomerase. Lanes 2 and 3: the reaction also contained 10 nM indicated protein and 5 mM ATP. (**C**) Quantification of the total DNA synthesis relative to synthesis in absence of hPif1. (**D**) Processivity analysis of telomerase in each reaction. (**E**) Unwinding of DNA/RNA hybrid by hPif1. The DNA substrate was linearized M13mp7 single-stranded DNA to which radiolabeled 36mer and 25mer RNA oligonucleotides had been annealed. The reaction with 50 nM recombinant protein is indicated on top of each lane. The products were examined by 12% native gel.

expressed hPif1 resets telomere length to the level of the starting population. These data argue that down-regulation of hPif1 expression promotes telomere elongation, and the telomere shortening caused by up-regulation of hPif1 expression is likely a direct effect.

hPif1 inhibits telomerase activity *in vitro*

Since overexpression of hPif1 causes telomere shortening, it is possible that the hPif1 inhibits telomerase activity. Therefore, we analyzed the effect of hPif1 on telomerase activity *in vitro* by using a PCR-based telomeric repeat amplification protocol (TRAP) assay. Telomerase-containing extracts were prepared from HT1080 cell lysate. In the first step of the reaction, telomerase adds a number of telomeric repeats (GGTTAG) on to the 3′ end of a substrate oligonucleotide. In the second step, the extended products are amplified by PCR, generating a ladder of products with six base increments on a native gel. With the increase of recombinant ^{GST}hPif1ΔN, telomerase ladders were decreased (Figure 5A, lanes 4–9). Interestingly, yPif1p showed a similar effect (Figure 5A, lane 10), suggesting that yPif1p and hPif1 are functional counterparts involved in the regulation of in telomere length (28,31,32). In contrast, ^{GST}hPif1^{K234A}ΔN lacked any detectable inhibitory activity (Figure 5A, lane 3). These data indicated that hPif1 inhibits telomerase activity *in vitro*.

hPif1 reduces processivity of human telomerase *in vitro*

Although the results of the TRAP assay (Figure 5A) suggested that hPif1 inhibits telomerase activity, it could not discern which aspect, i.e. the primer binding or processivity of telomerase is affected by hPif1. Processivity is defined as the ability of telomerase to undergo multiple reaction cycles without being released from telomeric DNA. To determine whether hPif1 affects primer binding or processivity of human telomerase, we performed direct telomerase activity assay, which allows us to assess telomerase processivity, i.e. the actual number of telomeric repeats added to the primer (telomerase ladder). When the recombinant ^{GST}hPif1ΔN was

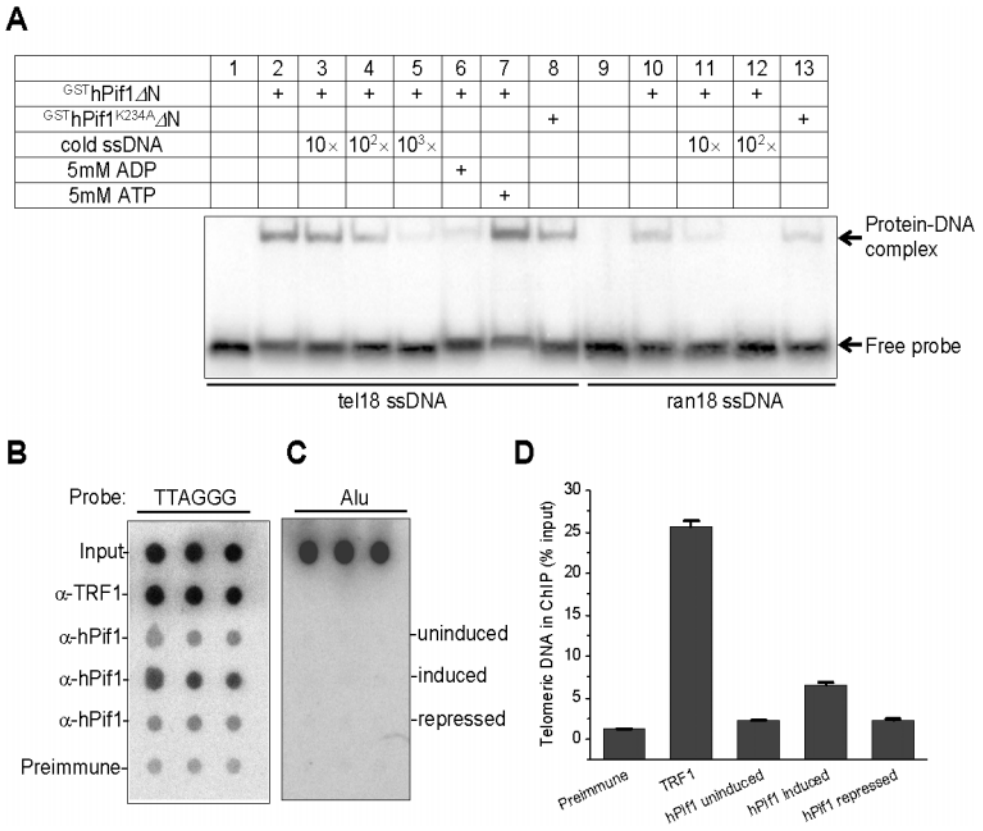

Figure 6. hPif1 binds telomeric DNA *in vitro* and *in vivo*. (**A**) Gel-shift assay of radiolabeled human telomeric DNA (tel18) or random single-stranded DNA (ran18) in presence or absence of 50 nM ^{GST}hPif1ΔN or ^{GST}hPif1^{K234A}ΔN. 10-, 100- and 1000-fold excess of unlabeled cold oligonucleotide was added as indicated. ATP or ADP was added after the reactions were incubated at 30°C for 30 min, and followed another 15 min incubation. The products were analyzed on 6% native gels. (**B**) Telomeric ChIP on HT1080 Tet-Off cell clones inducibly expressing hPif1^{myc} using the indicated antibodies or pre-immune serum. Dot blots were hybridized with a TTAGGG repeat probe. HT1080 Tet-Off cell clone T15 was induced to express hPif1 for 7 days and were processed alongside uninduced cells, and hPif1 expression was repressed after induction. Positive control is polyclonal anti-TRF1 antibodies (*ab1428*, Abcam). (**C**) Duplicate dot blots was probed for Alu repeats. (**D**) Quantification of the signals in (B). Averaged duplicate signals obtained with total DNA samples were used as 100% value for the quantification.

added into the telomerase reaction, the total DNA synthesis of telomerase was reduced (Figure 5B and C), arguing that hPif1 inhibits telomerase activity, which is consistent to the TRAP assay result. Strikingly, the presence of recombinant ^{GST}hPif1ΔN resulted in a significant decrease of the repeated ladders formed by multiple telomeric repeats addition (Figure 5B, lanes 1, 2 and Figure 5D), indicating that hPif1 dramatically reduces the processivity of human telomerase. In contrast, little difference could be seen when recombinant ^{GST}hPif1^{K234A}ΔN was added into the telomerase reaction (Figure 5B, lanes 1, 3 and Figure 5C and D), suggesting that ATPase/helicase activity is essential for the inhibitory effect of hPif1 on telomerase processivity.

hPif1 has the ability to dissociate RNA from DNA

We then investigated mechanism of the inhibition of telomerase by hPif1. Our initial suspicion was that hPif1 could dissociate telomerase RNA from its telomeric DNA. However, even though the helicase activity of Pif1 seems important for its function, but the activity for unwinding DNA/DNA with a 5′ to 3′polarity (Figure 2C) could not explain its inhibitory effect on telomere elongation because telomeres have 3′ single-stranded sequence. Therefore, we tested the activity of hPif1 using radiolabeled 25mer and 35mer RNA oligonucleotides annealed with M13mp7 single-stranded DNA. In the presence of either yPif1 or ^{GST}hPif1ΔN, the 35mer RNA oligonucleotide was released from the M13mp7 DNA (Figure 5C, lanes 2 and 4) (30), while helicase-deficient hPif1 or yPif1 did not show the unwinding activity (Figure 5C, lanes 3 and 5). Hence, we were able to conclude that hPif1 possesses ATP-dependent activity that unwinds a DNA/RNA duplex with 5′ to 3′polarity *in vitro*.

To investigate the possibility of a physical interaction between hPif1 and hTERT, we performed co-immunoprecipitation and the yeast two-hybrid assay. Both assays produced negative results, suggesting that hPif1 is unlikely to interact with hTERT (data not shown). Taken together, our findings suggest that hPif1 inhibits telomerase activity by unwinding the duplex of telomerase RNA-telomeric DNA hybrid.

hPif1 binds human telomeric DNA *in vitro* and *in vivo*

If the inhibition of hPif1 on telomerase is direct, it may interact with telomeric DNA. To test this, we carried out gel-shift assay of single-stranded human telomeric DNA or random DNA using purified recombinant ^{GST}hPif1ΔN or ^{GST}hPif1^{K234A}ΔN. Random single-stranded DNA was added as molecular competitor. The recombinant ^{GST}hPif1ΔN could

bind single-stranded DNA with either random or human telomeric sequence, but exhibited higher affinity for telomeric DNA because 100-fold more random DNA appeared not to be able to compete with telomeric DNA (Figure 6A, lanes 2 and 3). In addition, ATP enhanced, while ADP reduced the binding of hPif1 to telomeric DNA (Figure 6A, lanes 6 and 7). Interestingly, ATPase-deficient mutant hPif1 also bound to single-stranded DNA, although with a slightly lower affinity (Figure 6A, lane 8), implying that ATP acted as a positive modulator rather than being an absolute requirement for the binding process. These results indicate that hPif1 is a single-stranded DNA-binding protein and has a preference for TTAGGG telomeric repeat *in vitro*.

To address whether hPif1 interacted with telomeric DNA *in vivo*, we performed ChIP experiments with extracts prepared from the cells either before induction of hPif1, after induction of hPif1, or upon repression of induced hPif1. The presence of telomeric DNA was detected using a dot blot assay with a probe incorporating TTAGGG repeats (Figure 6B). The telomere-associated protein TRF1 was used as a positive control. We found that the affinity-purified anti-hPif1 antibodies pulled down hPif1 with associated telomeric DNA only when the expression of hPif1 was induced (Figure 6B). When a non-telomeric probe such as Alu DNA was used in the ChIP analysis, only the input extracts generated a positive signal (Figure 6C). These results suggested that hPif1 interacts with telomeric DNA *in vivo*, and that the inhibitory effect of hPif1 on telomere elongation might be direct.

DISCUSSION

In this work, we identified a human helicase hPif1. Like the yeast Pif1, hPif1 is able to unwind DNA/DNA (Figure 2C) and DNA/RNA duplex (Figure 5E), and is a putative homologue of *E.coli* RecD (Figure 1 and Supplementary Figure 1). Ectopic expression of wild-type, but not ATPase/helicase-deficient hPif1 causes telomere shortening in telomerase-positive cells, and repression of ectopically expressed hPif1 restored the equilibrium of telomere length (Figure 4). The hPif1 protein preferentially binds telomeric TTAGGG repeats (Figure 6A), and decreases telomerase processivity *in vitro* (Figure 5B–D). The inhibitory effect of hPif1 on telomere elongation is likely to be direct because hPif1 associates with telomeric DNA *in vivo* (Figure 6B). We propose that hPif1's ability to unwind the DNA/RNA duplex formed by telomerase RNA and telomeric DNA defines the mechanism whereby hPif1 inhibits telomerase activity (Figure 7).

Helicases are the motor proteins that utilize the energy of NTP hydrolysis to transiently catalyze the unwinding of duplex nucleic acids, and are found to be essential in many cellular processes involving DNA and RNA. Pif1 helicase subfamily belongs to the helicase superfamily I (SFI). However, when the members of Pif1 helicase subfamily were analyzed using the COG database, they were grouped in RecD COG, while WRN and BLM helicases fell into RecQ COG (Supplementary Table 1). We propose that hPif1and the other members of Pif1 subfamily are RecD-like helicases. The sequence alignment of conserved helicase motifs supports this hypothesis. In addition to the helicase motifs,

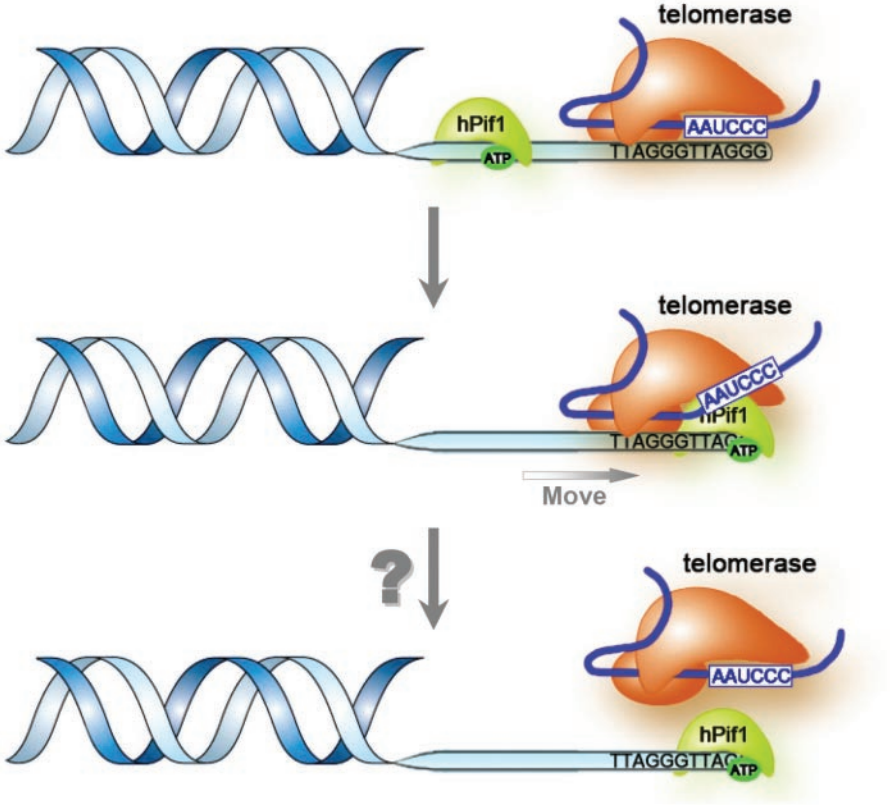

Figure 7. Model for hPif1 as a telomerase inhibitor. hPif1 dissociates telomerase RNA from telomeric DNA.

Pif1 subfamily and prokaryotic RecD subfamily share some conserved motifs, named A, B and C (Figure 1 and Supplementary Figure 1), which are not found in other members of SFI. Besides the sequence similarity, functional studies also strengthened this idea. RecD helicase in *E.coli* has been reported to form a complex with RecBC to repair DNA breaks by homologous recombination via a concerted action of helicase and nuclease activities (39). The yeast Pif1p has previously been shown to participate in mitochondrial DNA recombination (42). The *Saccharomyces* Rrm3p, which is a paralogue of Pif1p, is required to reduce the converged forks during rDNA replication (29). Thus, like RecD, the yPif1p and Rrm3p are involved in DNA recombination, and it remains a possibility that hPif1 also takes part in other cellular processes. Also of note, hPif1 does not appear to be the only RecD-like helicase in humans. The mammalian DNA helicase B, which is required for the onset of chromosomal DNA replication, shares homology with RecD (43) (Supplementary Table 1). Thus, it will be interesting to further study the functions of RecD-like helicase in mammals.

The members of Pif1 helicase subfamily studied so far all appear to contribute to telomere maintenance. In *S.pombe*, mutation of the Pfh1p results in modest telomere shortening (44). In *S.cerevisiae*, the Rrm3 helicase promotes the passage of the replication fork through telomere and sub-telomere regions (30). Pif1p inhibits telomere elongation by reducing the processivity of the telomerase and by removing the telomerase from telomere ends (31), In humans, overexpression of hPif1 causes telomere shortening (Figure 2B), and this negative regulation is most likely achieved through the inhibition of telomerase (Figure 5A–D). Repression of overexpressed hPif1 resulted in telomere elongation (Figure 2B). Therefore, the general inhibitory functions of hPif1 and yPif1p in telomeres are similar (31). However, the precise mechanisms of telomerase inhibition by hPif1 and yPif1p may not be identical: hPif1 appears to reduce the telomeric repeat addition processivity (Figure 5B–E), while yPif1p releases telomerase from telomeric DNA in an active form after completion of one round of DNA synthesis (31). In all cases, the effects of these proteins on telomeres require ATPase/helicase activity. These observations from studies *in vitro* and *in vivo* lead us to propose a model depicted in Figure 7. hPif1 binds a single-stranded G-rich region that is proximal to the double-stranded region of the telomere, while telomerase binds the region distal to single-stranded region and the telomerase RNA pairs with the tip of 3′ overhang (Figure 7). In this model, Pif1 would slide along the DNA in a 5′ to 3′direction to access and disentangle the telomerase RNA-telomeric DNA hybrid, thereby inhibiting telomere elongation. ATP and ADP might act as regulators of the process. When hPif1 binds ATP, it may undergo a conformational change that facilitates it's binding to ssDNA and provide the energy to move along the telomeric DNA. With the binding of ADP by hPif1, access to ssDNA may be restricted, or alternatively, it may be released from the telomeric DNA (Figure 7). However, the question of whether hPif1 is capable of directly removing hTERT from telomeric DNA *in vivo* needs further investigation.

The inhibition of telomere elongation as a result of DNA/RNA unwinding orchestrated by hPif1 represents a different regulatory mechanism for human telomerase from those previously proposed (19,20,27,44–46). The notion has emerged that helicases are required for telomere replication. Recent studies indicate that the *Saccharomyces* Sgs1 helicase (47–49) and human WRN helicase (50), two members of RecQ subfamily, are involved in telomere maintenance. However, unlike Pif1 helicase, which seems to fall in RecD subfamily and regulates telomere length through inhibition of telomerase, the Sgs1 helicase participates in a telomerase-independent pathway for telomere lengthening (48,49). The WRN helicase, in which a mutation causes the Werner syndrome, is required for dissociating alternate or secondary structures at telomere, to allow for replication, repair and telomerase activity at the telomere end (50,51). However, whether loss of function of hPif1 would correlate with any of human disease needs further investigation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Chantal Autexier for providing the phTERT and phTR+1 plasmid, Drs Mujun Zhao, Zhengjun Chen, Naihe Jing for providing reagents, and Dr Bibo Li for the protocols of telomere Southern blot and data quantification. The authors thank Drs Louis Carastro, Bibo Li and Peter Wookey for their critical reading. This work is supported by a Chinese Academy of Sciences-Max Planck Society Professorship, and grants from Commission of Science & Technology Shanghai Municipality (04DZ14006), Ministry of Science and Technology (2005CB522400), National Natural Science Foundation of China (NSFC 30125010 and 30270295). Funding to pay the Open Access publication charges for this article was provided by Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Conflict of interest statement. None declared.

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