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Research paper

The OB-fold domain 1 of human POT1 recognizes both telomeric and non-telomeric DNA motifs

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ABSTRACT

The POT1 protein plays a critical role in telomere protection and telomerase regulation. POT1 binds single-stranded 5′-TTAGGGTTAG-3′ and forms a dimer with the TPP1 protein. The dimer is recruited to telomeres, either directly or as part of the Shelterin complex. Human POT1 contains two Oligonucleotide/Oligosaccharide Binding (OB) fold domains, OB1 and OB2, which make physical contact with the DNA. OB1 recognizes 5′-TTAGGG whereas OB2 binds to the downstream TTAG-3′. Studies of POT1 proteins from other species have shown that some of these proteins are able to recognize a broader variety of DNA ligands than expected. To explore this possibility in humans, we have used SELEX to reexamine the sequence-specificity of the protein. Using human POT1 as a selection matrix, high-affinity DNA ligands were selected from a pool of randomized single-stranded oligonucleotides. After six successive rounds of selection, two classes of high-affinity targets were obtained. The first class was composed of oligonucleotides containing a cognate POT1 binding sites (5′-TTAGGGTTAG-3′). The second and more abundant class was made of molecules that carried a novel non-telomeric consensus: 5′-TNCANNAGKKKTTAGG-3′ (where K = G/T and N = any base). Binding studies showed that these non-telomeric sites were made of an OB1-binding motif (TTAGG) and a non-telomeric motif (NT motif), with the two motifs recognized by distinct regions of the OB1 domain. POT1 interacted with these non-telomeric binding sites with high affinity and specificity, even when bound to its dimerization partner TPP1. This intrinsic ability of POT1 to recognize NT motifs raises the possibility that the protein may fulfill additional functions at certain non-telomeric locations of the genome, in perhaps gene transcription, replication, or repair.

1. Introduction

Telomeres are essential structures that cap and protect the ends of linear chromosomes [1,2]. A key function of telomeres is to hide chromosomal ends from DNA damage sensing mechanisms that would otherwise recognize them as double-stranded DNA breaks. A loss of this function elicits a DNA damage response that results in the induction of senescence or apoptosis. This uncapping of telomeres can also lead to cellular attempts to repair the ends using non-homologous end-joining (NHEJ), a process that generates telomere–telomere fusions and dicentric chromosomes. In humans, telomeres are made of tandem $(TTAGGG)_n$ DNA repeats, which are made by the enzyme telomerase [3,4]. The bulk of the telomere is composed of duplex telomeric DNA, except for the last 50–400 bases of the G-rich strand that give rise to a single-stranded 3′-overhang [1,2]. Evidence suggests that this telomeric 3′-overhang is sequestered into a large lariat structure, termed a T-loop [5,6]. Formation of this structure involves the insertion of the telomeric 3′-overhang into duplex telomeric DNA and its hybridization to the C-rich strand, thereby displacing the opposite G-rich strand to form a displacement loop (D-loop). It has been proposed that these T-loops are especially well-adapted to shield the

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chromosomal ends from DNA repair and DNA damage sensing mechanisms [5,6]. Even more critical for telomere capping and telomerase regulation are the protein complexes associated with telomeres, in particular the Shelterin complex and POT1/TPP1 dimer [5,7]. Common to both complexes is the POT1 protein (Protection of Telomere 1).

The human POT1 protein is a telomere-associated factor that binds selectively and with high affinity to single-stranded 5′-TTAGGGTTAG-3′ [8–10]. POT1 binding sites are potentially present at the telomeric 3′-overhangs, D-loops, and in DNA bubbles that may form in duplex telomeric DNA as a result of G-quadruplex formation and/or activities of DNA helicases. An important role played by the POT1/TPP1 dimer is to prevent recognition of the telomeric 3′-overhang as a single-stranded gap of unreplicated or otherwise damaged DNA [11–14]. These gaps in the genome are detected by the sequence-independent ssDNA-binding Replication Protein A (RPA) complex in conjunction with the 9-1-1 complex (Rad9-Hus1-Rad1). The recruitment of these complexes to a single-stranded gap results in the activation of the ATR kinase, formation of a DNA damage foci, and inhibition of cell cycle progression [15,16]. The higher affinity of the POT1/TPP1 dimer for telomeric 3′-overhangs is sufficient to block the recognition of these sites by the more abundant RPA and 9-1-1 complexes. Hence, the loss of POT1 results in the telomere localization of RPA, activation of the ATR kinase, and formation of telomere dysfunction-induced foci (TIF) [11–14].

A second key function of the POT1/TPP1 dimer is to regulate telomerase at individual telomeres, both positively and negatively. First and foremost, the dimer provides a docking site for the recruitment of telomerase to the telomeric 3′-overhang. TPP1 physically interacts with the catalytic subunit of telomerase (hTERT), and this interaction is needed for the telomere localization of telomerase [17,18]. When bound to telomerase, the POT1/TPP1 dimer also increases the processivity of the enzyme, enabling it to make additional repeats in a single binding event [19,20]. The POT1/TPP1 dimer can also function as a negative regulator of the access of telomerase to telomeres – a function that the dimer accomplishes as part of the multi-component Shelterin complex. In the Shelterin, the POT1/TPP1 dimer is combined with telomeric factors that recognize duplex telomeric DNA: the TRF1 dimer and TRF2/RAP1 tetramer [7,21–23]. A scaffolding subunit, TIN2, interacts simultaneously with POT1/TPP1, TRF1 dimer, and TRF2/RAP1 complex to form the Shelterin complex [21,24–30]. At telomeric dsDNA/ssDNA junctions, the complex can interact simultaneously with duplex telomeric DNA (via TRF1 and/or TRF2) and with the telomeric 3′-overhang (via POT1). At this site, the complex is ideally positioned to restrict the availability of the 3′-overhang for elongation by telomerase. The complex is a negative regulator of telomerase, and its loss in telomerase-expressing cells results in a deregulated lengthening of the telomeres [12,23–27,29,31–34].

The POT1/TPP1 dimer relies on POT1 for DNA binding, as the TPP1 protein lacks DNA-binding activity. The minimum tight-binding telomeric binding site for human POT1 is 5′-TTAGGGTTAG-3′, which the protein recognizes by means of two OB-fold domains, OB1 and OB2 [10]. The OB-fold is a five-stranded anti-parallel β-barrel that mediates protein–protein interactions and ssDNA binding. In human POT1, the DNA-binding grooves of OB1 and OB2 form a single continuous channel for the telomeric ssDNA. OB1 recognizes the 5′-TTAGGG segment whereas OB2 associates with the downstream TTAG-3′ [10]. Upon binding, the DNA adopts an extended conformation with its backbone exposed to the solvent and its bases buried in the groves. The hydrogen bonds between the bases and side chains of the DNA-binding grooves dictate the sequence specificity of the protein [9,10], with the majority of these interactions involving OB1 (22/31 hydrogen bonds). The protein/DNA complex is further stabilized by hydrophobic interactions between stacked pairs of adjacent bases (T1/T2, A3/G4, G5/G6, and T8/A9) and aromatic side chains. These hydrophobic interactions help stabilize the complex but contribute little to sequence specificity [8,10,35].

Similar to human POT1, the fission yeast POT1 protein contains two OB-fold domains (Pot1pN and Pot1pC) that mediate ssDNA-binding. Recent studies have revealed an unexpected capability of Pot1pC to recognize a variety of non-cognate ligands [36,37]. Surprisingly, the non-cognate ligands were recognized through hydrogen bond interactions that typically contribute to sequence specificity. The broad DNA specificity was derived from local reorientations of bases and/or side chains or by global conformational changes affecting both protein and DNA [36,37]. Whether the human POT1 protein exhibits a similar flexibility and is able to interact with non-telomeric ligands had not yet been formally investigated. To address this possibility here, we have used SELEX (Systematic Evolution of Ligands through Exponential Enrichment) to reexamine the DNA-binding specificity of human POT1.

In SELEX, the protein of interest is used as a selection matrix to capture high-affinity ligands from a pool of random DNA molecules [38,39]. By the successive capture of POT1/DNA complexes, two classes of high-affinity POT1 ligands were identified from a pool of randomized ssDNA molecules by SELEX. One class corresponded to the cognate telomeric binding site of human POT1 made of juxtaposed OB1- and OB2-binding motifs (5′-TTAGGGTTNG-3′, where N is any nucleotide). The second, more abundant class instead combined an OB1-binding motif (5′-TTAGGN-3′) with a novel non-telomeric DNA motif (5′-TNCAN-NAG-3′). *In vitro* binding studies confirmed the high affinity interaction of human POT1 with these non-telomeric binding sites. This capability of human POT1 to recognize non-telomeric sequences raises the possibility that the protein might play additional roles at non-telomeric locations, in perhaps gene transcription, replication, or repair.

2. Materials and methods

2.1. Materials

Oligonucleotides were synthesized by the Eppley Core Facility (University of Nebraska Medical Center, Omaha, NE). The polynucleotide kinase and the Pfx and Taq DNA polymerases were purchased from Invitrogen (Carlsbad, CA). All other enzymes were obtained from Fermentas (Hanover, MD), New England BioLabs (Beverly, MA), Promega (Madison, WI) or Invitrogen (Carlsbad, CA). Protease inhibitor cocktails for bacterial extracts (cat # P8849) and mammalian extracts (cat # P8340) were purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals were obtained from ThermoFisher Scientific (Waltham, MA). The TnT® Quick Coupled Transcription/Translation System was from Promega (Madison, WI). The γ-[^{32}P]-ATP (4500 Ci/mmol) was from MP Biologicals (Solon, OH). M450 magnetic beads coated with a sheep anti-mouse IgG antibody were from Dynal Biotech. Inc. (Lake Success, NY). Mouse monoclonal antibody against the Flag tag (IgG_1 clone M2) was purchased from Sigma–Aldrich (St. Louis, MO) and the normal mouse IgG (cat # sc-2025) was obtained from Santa Cruz (Santa Cruz, CA). Rabbit polyclonal antibody against amino acids 250–350 of human POT1 was from Abcam (Cambridge, MA). The purified recombinant human Flag-POT1 (cat # TP316275) and Flag-TPP1 (cat # TP304381) proteins were obtained from OriGene Technologies (Rockville, MD).

2.2. Expression vectors

Expression vector pCMV1-Flag-POT1 was made in a three step process. First, an EcoRI/PstI fragment encoding the C-terminal half of human POT1 was excised from plasmid pOTB7-POT1 (BC002923; Open Biosystem, Huntsville, AL) and inserted in the EcoR1/PstI sites of the pCMV1-Flag vector. The pCMV1-Flag plasmid contains CMV/T7 promoters driving the expression of a cassette encoding the Flag epitope (DYKDDDDK) and followed by a unique EcoRI site. Second, an EcoRI-digested PCR fragment carrying the N-terminus of POT1 was inserted at the EcoRI site that separates the Flag tag and C-terminus of POT1. This fragment was PCR amplified from pOTB7-POT1 using a 5′-CCGGAATTCGGTGGAATGTCTTTGGTTCCAGCAA-CAAATT-3′ primer (EcoRI site and initiation codon underlined) and a T7 primer. Finally, the resulting construct was cut with SspI and EcoRV and self-ligated to delete the additional T7 promoter, which had been introduced as part of the initial pOTB7-POT1 fragment (Step 1).

pcDNA3.1 expression vectors were also made for each of the Flag-POT1 mutants. These vectors were made by the insertion of a PCR product carrying a blunt end and an XbaI cohesive end into the EcoRV and XbaI sites of the pcDNA3.1(+) vector. Using plasmid pCMV-Flag-POT1 as template, PCR products were amplified with Pfx using the forward primer 5′-TCTGCTAGCATGGACTACAAAGACGATGACGAC-3′ (ATG of Flag tag underlined) and one of the following reverse primers: 5′-GAC**TCTAGA**TTAATCACACAATTTTAGTAATGTCCAAGAC-3′ (to make pcDNA3.1-Flag-POT1(1–155); XbaI site bolded; termination codon underlined), 5′-GAC**TCTAGA**TTAATGATCTGTAAGTATTGTAGCAGA (to make pcDNA3.1-Flag-POT1(1–340); XbaI site bolded; termination codon underlined), or 5′-GAC**TCTAGA**TTAAAACTTGTTCGAGAGTTT GCAAAT-3′ (to make pcDNA3.1-Flag-POT1(1–470); XbaI site bolded; termination codon underlined).

Bacterial vector pET28a-POT1(1–314) expressing a His-tagged human POT1(1–314) protein was made by insertion of a FauI/SalI-digested PCR product into the NdeI/SalI sites of pET28a(+). This product encoding human POT1(1–314) was amplified with Pfx using pCMV-Flag-POT1 as template. Primers used for the PCR amplification were as follows: 5′-GCTCTACCCGCTACATATGTCTTTGGTT-CCAGCAACA-3′ (as the forward primer; FauI site underlined) and 5′-GCTGAGGTCGACTAGCTGTCGTCAGGTTCTGATTGAC-3′ (as the reverse primer; SalI site underlined).

2.3. In vitro transcription/translation of the Flag-POT1 proteins

The Flag-POT1 proteins were produced by *in vitro* transcription/translation in a rabbit reticulocyte lysate. Flag-tagged version of full length POT1 and each POT1 mutants were synthesized as directed by the pCMV1-Flag-POT1 vector (full length) and pcDNA3.1-Flag-POT1 vector series (aa 1–155, 1–340, 1–470), respectively. In a final volume of 50 μl, 1 μg of POT1 plasmid was transcribed/translated using the TnT® Quick Coupled system, according to the manufacturer's instructions (Promega, Madison, WI). A water-programmed lysate (Mock) was produced in parallel to serve as a negative control. Aliquots of the reactions were analyzed by Western blotting using the anti-Flag M2 antibody (Fig. 4B).

2.4. Flag-POT1 in extracts of transfected 293T cells

293T cells were cultivated in 5% CO_2 at 37 °C in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. Log growth cells were transiently transfected by the calcium phosphate method using MBS mammalian transfection kit (Stratagene, La Jolla, CA), following the manufacturer's instructions. Cells were transiently transfected without plasmid (Mock) or with the pCMV1-Flag-POT1 vector (Flag-POT1). Forty-eight hours post-transfection, cells were harvested for preparation of whole cell extracts, as described previously for the Shelterin complex [40].

2.5. His-tagged human POT1(1–314) in extracts of Escherichia coli cells

Bacterial vector pET28a-POT1(1–314) expressing a His-tagged human POT1(1–314) was transformed into Rosetta2(DE3) cells (Novagen) following standard protocols. Cells were grown at 37 °C until reaching an OD_{600} of 0.5, after which cells were induced with 1 mM IPTG at 37 °C for 4 h prior to harvesting. The cell pellet was lysed in buffer A (20 mM BICINE, 100 mM NaCl, 40 mM imidazole, 2 mM β-mercaptoethanol, pH 8.5) plus protease inhibitor cocktail (Sigma, #P8849) using three passes at 15,000–20,000 psi in an Emulsiflex C-5 (Avestin Inc., Ottawa, ON). The extract was cleared by centrifugation at 18,000 g for 30 min. The His-tagged POT1(1–314) protein was detected by Western blot using a nickel (Ni^{2+})-activated derivative of horseradish peroxidase, HisProbe-HRP (ThermoFisher Scientific, Waltham, MA). Detection was following the manufacturer's instructions, except that the blocking and binding steps were performed in the presence of 3 M guanidine-HCl.

2.6. SELEX

SELEX was performed as we have previously done [41], except for minor modifications. A 75-mer single-stranded (ss) oligonucleotide containing a 35-nt random core flanked by PCR priming sequences (Fig. 1A) was used as starting material. In the first round of selection, 5 μg of this randomized oligo (215 pmol, 130 trillion molecules) was incubated with 5 μl of rabbit reticulocyte lysate programmed with Flag-POT1 in a 20 μl reaction containing 1× binding buffer (4% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5). After 30 min at room temperature, 10 μl of magnetic beads coated with the anti-Flag M2 antibody were added. M450 Sheep anti-Mouse IgG beads coated with the M2 antibody were prepared as previously described [40,42]. After an hour of rotation at 4 °C, the beads were washed 3 times with 500 μl of ice-cold 1× binding buffer, after which the selected oligos were eluted at 95 °C for 5 min in a 100 μl of PCR mix. Re-amplification of the eluted molecules was done with Taq DNA polymerase using primers 5′-CAGTAGCACACGACATCAAG-3′ (forward) and 5′-CAACTGACACGAGACATGCA-3′ (reverse). Aliquots taken after 10, 15, 20 and 25 cycles of PCR were analyzed by electrophoresis on a 3% agarose gel. The most optimally amplified DNA product (no smear, no supershift, and within the exponential range) was excised and gel purified using the GENECLEAN kit (MP Biologicals, Solon, OH). To regenerate the ssDNA molecules needed for the next round of SELEX, the isolated DNA was subjected to 16 cycles of asymmetric PCR with Taq DNA polymerase and the forward primer alone. After extraction with phenol: chloroform (1:1) followed by chloroform alone, the ssDNA molecules were ethanol precipitated and subsequently dissolved in 1× binding buffer for the next round of SELEX. Additional rounds of SELEX were performed identically to the first round, except that the input DNA was made of the previously selected and reamplified ss-oligonucleotides supplemented with 5 μg of sonicated salmon sperm DNA. After the sixth round of selection, the reamplified and gel purified double-stranded DNA was cloned into TA-cloning vector pCR2.1-Topo following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Chemically transformed TOP10 *E. coli* cells were plated in the presence of kanamycin (25 μg/ml). A total of 50 colonies were picked and analyzed by sequencing.

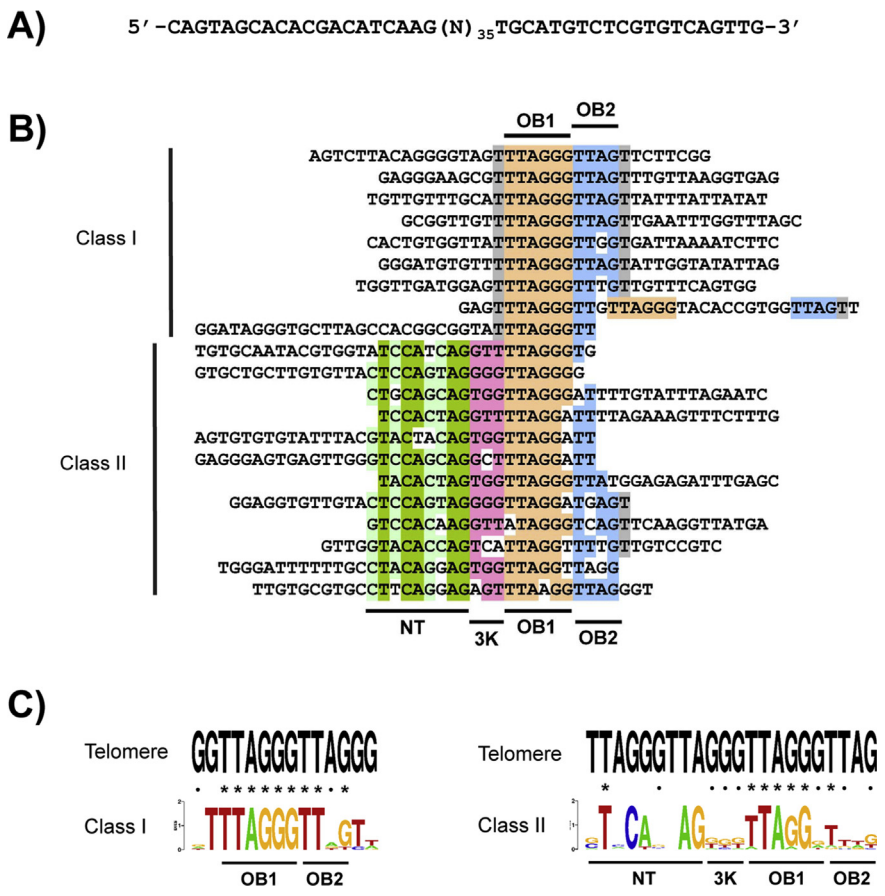


Fig. 1. SELEX identifies POT1 binding sites comprised of telomeric and non-telomeric DNA motifs. A) Sequence of the randomized oligonucleotide library used for SELEX. The random core of 35 nucleotides is flanked by PCR priming sequences. B) Sequences of the oligonucleotides selected by the Flag-POT1 protein after six consecutive rounds of SELEX. Only the sequences of the 35 nucleotides random cores are shown. Two classes of binding sites (Classes I and II) were identified based on the presence or absence of the NT motif (green). Also noted in the selected random cores were a 3K motif (purple), OB1 motif (orange), and OB2 motif (blue). C) Sequence logo representations of the consensus of class I (top panel) and II (top panel) binding sites, and their comparison with the sequence of human telomeres. Star and dot denotes completely and partially conserved matches, respectively. Sequence logos were generated by the WebLogo program.

2.7. Western blotting

Proteins were quantified using the Bradford's assay (Bio-Rad). Samples (80–100 μg/well) were separated on 4–20% gradient SDS-PAGE gels (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). Blocking steps and incubations with the antibodies were done in TBS-T (50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6) containing 5% fat-free dry milk. Washes were done using TBS-T. Signals were detected using the SuperSignal West Pico kit (ThermoFisher Scientific, Waltham, MA). Antibodies used included the M2 mouse monoclonal antibody (Sigma–Aldrich, St. Louis, MO) and rabbit polyclonal antibody against amino acids 250–350 of human POT1 (Abcam, Cambridge, MA). Secondary antibodies used were horseradish peroxidase-conjugated antibodies against mouse or rabbit IgG (Jackson ImmunoResearch, West Grove, PA).

2.8. Silver staining

SDS-PAGE gel was fixed for 90 min in an aqueous solution containing 50% methanol (v/v), 12% acetic acid (v/v), and 1.88% formaldehyde (w/w). After washing the gel 3 times for 10 min each in the same solution without formaldehyde, the washes were repeated with distilled water. The gel was treated with 0.02% sodium thiosulfate for 2 min, washed in distilled water (3 times, 30 s each), and then exposed to silver nitrate (0.2% silver nitrate, 0.0188% formaldehyde) for 30 min. The gel was transferred to a new container, washed with distilled water (3 times, 1 min each), and then developed in a solution containing 3% sodium carbonate, 0.0002% sodium thiosulfate, and 0.0188% formaldehyde. Development was quenched in the fixing solution without formaldehyde. Stained gels were photographed and then stored at 4 °C in a 1% acetic acid solution.

2.9. Electrophoretic mobility shift assay (EMSA)

In a final volume of 25 μl, binding reactions contained binding buffer (4% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5), 2 μg of denatured sonicated *E. coli* DNA, and 5 μl of a POT1 protein source (rabbit reticulocyte lysate, *E. coli* cell extract, or extract of transfected 293T cells). Binding reactions with the purified recombinant human Flag-POT1 protein (OriGene Technologies, 22 ng/reaction) were performed as above, except that a modified binding buffer was used to minimize protein aggregation (4% glycerol, 1 mM $MgCl_2$, 5 mM DTT, 100 mM NaCl, 10 mM Tris–HCl, pH 8.0). After 5 min at room temperature, 5–10 × 10^4 cpm of radiolabeled probe was added to each binding reaction. Radiolabeling of the probes was done as previously described [42]. Reactions were incubated for an additional 25 min, after which point protein/DNA complexes were resolved by native gel electrophoresis on a 4% polyacrylamide gel containing TBE (45 mM Tris-borate, 2 mM EDTA, pH 8.3) buffer. When performing supershifts, antibodies were always added for the last 5 min of

incubation, prior to electrophoresis. Gels were run at 180 V for 2 h at 4 °C. Gels were subsequently transferred to a DE81 anion exchange chromatography paper (Whatman International, Maidstone, England), dried, and exposed to a PhosphoImager screen to be imaged by audioradiography. In binding reactions containing the purified human POT1 and/or TPP1 proteins (both from OriGene Technology), a fixed amount of POT1 (0.5 pmol) was incubated with different molar ratios of TPP1 (0.5, 1, 2, or 4 pmol) at room temperature for 10 min prior to the addition of the radiolabeled probe.

2.10. Binding competition and determination of IC_{50}

For determination of IC_{50}, binding reactions were performed as described above (EMSA) with *in vitro* translated Flag-POT1, except that the [^{32}P]-labeled OB1/OB2 probe (2 nM) was added along with the indicated amounts of each of the unlabeled competitors. Following exposure to a PhosphoImager cassette, volume integration of EMSA signals was performed using the ImageQuant 5.2 software (Amersham Biosciences Corp., Piscataway, NJ). Signals were plotted as a function of log[C] (where C is the competitor concentration) and modeled to a sigmoidal dose–response curve using SigmaPlot 8.0 (SPSS Inc., Chicago, IL). For each competitor, an IC_{50} was calculated by mathematical regression, as well as a 95% confidence range associated with each value.

3. Results

3.1. SELEX identifies POT1 binding sites made of telomeric and non-telomeric DNA motifs

SELEX was used to reexamine the DNA-binding specificity of human POT1. An oligonucleotide library containing a random core of 35 nucleotides flanked by PCR priming sequences was synthesized (Fig. 1A). A Flag-tagged human POT1 protein was produced by *in vitro* transcription/translation in a rabbit reticulocyte lysate (Fig. 4B). The oligonucleotide library was incubated with the Flag-POT1 programmed reticulocyte lysate to allow for the formation of protein/DNA complexes. With beads coated by the anti-Flag M2 antibody, protein/DNA complexes containing Flag-POT1 were captured along with their associated DNA. The recovered ssDNA molecules were then PCR amplified, converted back to ssDNA, and subjected to additional rounds of selection. After six consecutive rounds of SELEX, the selected and reamplified DNA was cloned and sequenced (Fig. 1B).

Sequence analysis of the consecutively selected oligos revealed two classes of molecules (Fig. 1B). Class I binding sites (n = 9) shared an invariant 5′-TTTAGGGTT motif followed by a partially conserved AGT-3′ motif, which together formed a 5′-TTTAGGGTTNGT-3′ consensus (Fig. 1C, top panel). This consensus was interpreted as representing a canonical POT1 binding site (5′-TTAGGGTTAG-3′) flanked on both sides by a thymidine. Canonical POT1 binding sites, as previously described [10], are composed of juxtaposed elements recognized by the OB1 (5′-TTAGGG) and OB2 (TTAG-3′) domains of POT1. Class II binding sites (n = 12) were the most abundantly selected sequences by the Flag-POT1 protein (Fig. 1B). These molecules shared an even larger consensus composed of both telomeric and non-telomeric sequence motifs: TNCANNAGNNNTTAGGNT (Fig. 1C, bottom panel). This consensus was interpreted as being composed of four juxtaposed elements: a non-telomeric motif (motif NT: STNCANNAG, where S = C/G), a block of three G or T (motif 3K: KKK, where K = G/T), an OB1-binding motif (motif OB1: TTAGGR, where R = A/G), and a poorly conserved OB2-binding motif (motif OB2: TNNK, where K = G/T). Three of these elements occur naturally at the telomeres in the same order as they occur in class II binding sites (3K-OB1-OB2). In contrast, the NT motif is not expected to occur at any of the known in vivo binding sites of POT1, namely the telomeric D-loop and 3′-overhang.

3.2. Binding of POT1 to class II binding sites is dependent on the integrity of the NT motif

A major difference between the class I and II binding sites was the presence in the latter of the NT motif. A second major difference was the lack of an OB2-binding motif in the majority of class II molecules. Electrophoretic mobility shift assays (EMSA) were performed to investigate the significance of these differences. Radiolabeled probes that differed in the presence or absence of the NT and/or OB2 motifs were made (Fig. 2A).

Five of these probes were initially tested for their binding to an *in vitro* translated Flag-POT1 protein (Fig. 2B). Probes were incubated with rabbit reticulocyte lysates programmed with the Flag-POT1 vector (Flag-POT1) or with no plasmid (Mock). The prototypical class I probe carrying adjacent OB1 and OB2 binding motifs (probe OB1/OB2) interacted with the Flag-POT1 protein to form a detectable complex (arrowhead). This complex was produced by the Flag-POT1 lysate but not by the mock lysate (lanes 1 versus 6). As expected, the loss of the OB2 motif prevented recognition by POT1 of a probe containing an OB1 motif only (probe OB1). Yet, adding the NT motif upstream of this single OB1 motif was sufficient to reestablish binding to Flag-POT1 (probe NT/OB1). Neither the OB1 motif alone (probe OB1) nor the NT motif alone (probe NT) were sufficient for POT1 binding, but the two motifs together synergized to allow for the formation of a stable complex with POT1 (probe NT/OB1; lanes 2 and 5 versus lane 3). But when the highly conserved CANNAG consensus of the NT motif was altered, POT1 binding to the combined motifs was greatly reduced (probe nt/OB1). Taken together, these results show that an OB1 motif alone is insufficient for stable binding to POT1 unless it is combined with either an OB2 motif or an NT motif. These findings validate the results of the SELEX and provide an explanation for the retrieval of the two classes of binding sites (OB1/OB2-like class I sites; NT/OB1-like class II sites).

Similar conclusions were drawn from validation studies performed with a Flag-POT1 protein produced in transfected human cells (Fig. 2C). Extracts were prepared from HeLa cells that had been either transfected with Flag-POT1 or mock transfected cells. Incubation of probe OB1/OB2 with the Flag-POT1 extract, but not the mock extract, led to the formation of a detectable protein/DNA complex (lane 1, arrowhead), which could then be supershifted by the anti-Flag M2 antibody (lane 12, arrow). Again, the Flag-POT1 protein did not interact with probes containing the OB1 motif alone (probe OB1) or NT motif alone (probe NT). Yet, the protein strongly interacted with probe NT/OB1 containing the combined NT and OB1 motifs. Interestingly, an even higher binding activity was observed when the three motifs were combined together in a single probe (probe NT/OB1/OB2). This last result show that while the NT and OB2 motifs can both synergize with an OB1 motif to enhance POT1 binding, the two motifs are non-redundant and act independently.

To assess the relative contribution of the NT, OB1, and OB2 motifs in POT1 binding, competition binding assays were performed with the *in vitro* translated Flag-POT1 protein (Fig. 3). In these experiments, the oligonucleotides were compared for their ability to compete with a radiolabeled OB1/OB2 probe for binding to Flag-POT1 (Fig. 3A). Quantification of EMSA signals allowed for the determination of an IC_{50} for each competitor as a surrogate measure of the relative affinity of the POT1 protein for each probe (Fig. 3B). As shown in Fig. 3C, both the OB1/OB2 and NT/OB1 oligonucleotides were very effective competitors, whereas as the NT, OB1 and NT/OB2 were not. Interestingly, the NT/OB1/OB2 oligo was a more effective competitor than the NT/OB1 and OB1/OB2 oligos.

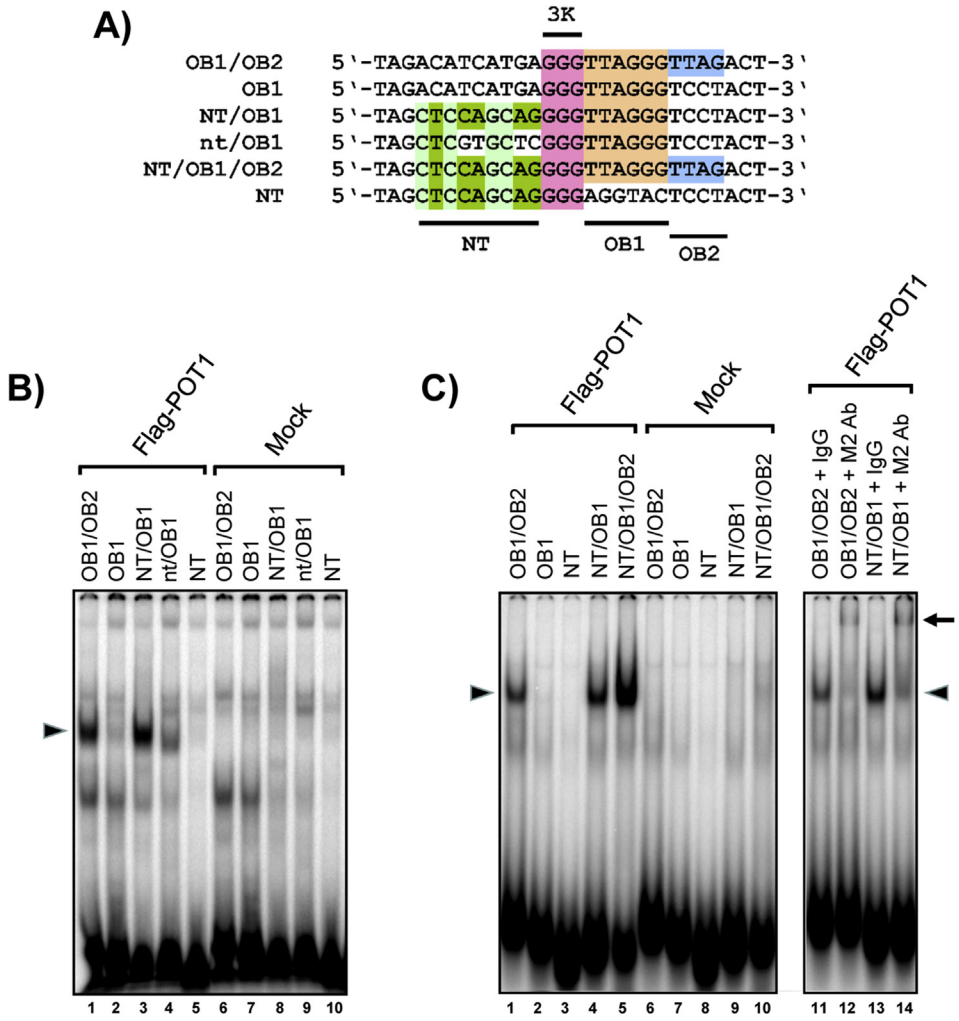


Fig. 2. The NT and OB1 motifs synergize to promote their recognition by human POT1. A) Graphical representations and sequences of the probes used for EMSA. All probes were labeled at the 5′-end with [^{32}P]. B) EMSA with an *in vitro* translated Flag-POT1 protein. The indicated probes were incubated with rabbit reticulocyte lysates programmed with the Flag-POT1 vector (Flag-POT1) or with no plasmid (Mock). The resulting protein/DNA complexes were resolved by electrophoresis in a native polyacrylamide gel. Arrowhead denotes position of the Flag-POT1/DNA complex. C) EMSA with Flag-POT1 present in extracts of transfected HeLa cells. HeLa cells transfected with the Flag-POT1 vector (Flag-POT1) or with no plasmid (Mock) were lysed and cleared by centrifugation. Extracts were incubated with the indicated probe, and protein/DNA complexes were resolved by EMSA. Arrowhead denotes the position of the Flag-POT1/DNA complexes. In lanes 11–14, antibodies were added in the last 5 min of incubation before electrophoresis, either the anti-Flag M2 antibody (M2) or pre-immune mouse IgG (IgG). Arrow denotes the position of the supershifted Flag-POT1/DNA complexes.

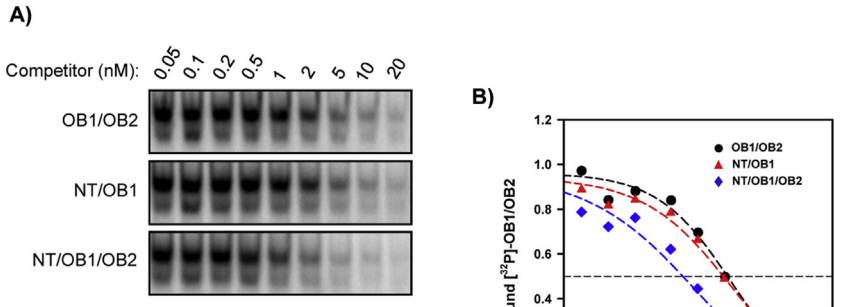
Again, these results imply that the OB2 and NT motifs are non-redundant and therefore must engage different DNA-binding determinants on the surface of POT1.

3.3. The OB1 domain of POT1 is responsible for the recognition of the NT motif

In the next series of experiments, we sought to identify the region of POT1 responsible for recognition of the NT motif. Truncated mutants of POT1 were created by deletion of different C-terminal segments of Flag-POT1 (Fig. 4A). The mutants were produced by *in vitro* translation and were tested for binding to a panel of 4 probes: OB1, NT, NT/OB1, and OB1/OB2 (Fig. 4B). Except for the POT1(1–470) protein, all other POT1 proteins exhibited DNA-binding activity. The full-length POT1 protein, POT1(1–634), interacted with both the NT/OB1 and OB1/OB2 probes, but not with the OB1 or NT probes. The POT1(1–340) mutant, which lacks the TPP1-interaction domain, behaved similarly and interacted with the NT/OB1 and OB1/OB2 probes. The POT1(1–155) mutant, which also lacks the OB2 domain, could no longer bind the OB1/OB2 probe but could still interact with the NT/OB1 probe. The POT1(1–155) mutant contains the OB1 domain only, which therefore indicates that this domain alone is sufficient for recognition of the NT motif and association with the NT/OB1 probe. The failure of the 1–155 mutant to recognize the OB1/OB2 probe was expected, as both the OB1 and OB2 motifs were shown to be required for recognition of telomeric POT1 binding sites [10]. Fig. 4C summarizes these results and shows that the OB1 domain is responsible for recognition of both the NT and OB1 motifs whereas the OB2 domain recognizes the OB2 motif. We also tested an untagged POT1 protein lacking a Flag epitope (Supplemental Fig. S1). This protein also interacted with the NT/OB1 probe, thereby showing that the Flag epitope is dispensable for recognition of the NT motif.

3.4. The NT motif interacts directly with POT1 in the absence of associated factors

The recognition of the NT motif might occur through direct physical interactions with the OB1 domain or alternatively, might be mediated by an interacting partners that binds to both the NT motif and OB1 domain of POT1. To distinguish between these



C)

Competitor	IC_{50} (nM)	IC_{50} range (nM)
OB1	> 20	N/A
NT	> 20	N/A
OB1/OB2	2.3	1.8 - 3.0
NT/OB1	2.2	1.6 - 3.0
NT/OB1/OB2	0.8	0.6 - 1.1

Fig. 3. Competitive binding assays and relative affinity of classes I and II sites for POT1. A) Competitive binding assays. Rabbit reticulocyte lysates programmed with Flag-POT1 were incubated with [^{32}P]-OB1/OB2 probe in the presence of a varied concentrations of each of the unlabeled competitors (OB1/OB2, NT/OB1, NT/OB1/OB2, NT, and OB1). Flag-POT1/DNA complexes were resolved by EMSA and the amount of radioactivity in the complexes was quantified by the ImageQuant program. B) Competition binding curves. Fraction of the [^{32}P]-OB1/OB2 probe bound to Flag-POT1 as a function of the concentration of each competitor. C) IC_{50} of each competitor. Calculated value (nM) and 95% confidence interval is shown for each. IC_{50} above 20 nM could not be reliably extrapolated. N/A: non-applicable.

possibilities, DNA-binding experiments were performed with additional sources of POT1 protein.

In Fig. 5A, an HIS-tagged POT1(1–314) protein was produced in *E. coli* cells, an organism that do not possess telomeres or POT1 orthologues. If the NT motif is recognized by an associated human or rabbit protein, then this factor is unlikely to be present in extracts of *E. coli*. The truncated POT1 protein was designed to contain the OB1 (aa 9–141) and OB2 (aa 161–278) domains but lacked the TPP1-interacting domain of POT1. In these experiments, expression of the His-tagged POT1(1–314) protein was induced or not by the addition of IPTG, and bacterial cell extracts were made and tested for DNA-binding. As the results show, IPTG induced the expression of the His-tagged protein (Fig. 5A) and of its DNA-binding activity (Fig. 5B). Most importantly, this DNA-binding activity had the same sequence specificity as that of the *in vitro* translated Flag-POT1 protein. Similar to the *in vitro* translated protein, the bacterially-produced His-tagged POT1 protein interacted strongly with both the OB1/OB2 and NT/OB1 probes (Fig. 5B). Again, binding to the NT motif itself or OB1 motif alone was limited, if not undetectable. Yet, the two motifs synergized to produce probe NT/OB1, the highest-affinity POT1 ligand tested.

In the next series of experiments, we tested a human POT1 protein purified to homogeneity from transfected human cells (OriGene Technologies Inc., Rockville, MD). Silver staining of the purified POT1 protein showed no other detectable proteins or contaminants, including the absence of TPP1 (Fig. 6A). Similar to the *in vitro* translated POT1 protein, the purified POT1 protein recognized both the OB1/OB2 and NT/OB1 probes (Fig. 6B). Again, the NT motif itself and OB1 motif alone provided little to no binding. Yet, the two motifs synergized in probe NT/OB1 to produce one of the highest-affinity POT1 ligands identified. These results show that the recognition of the NT motif by POT1 does not require the presence of additional proteins. Hence, we conclude that the NT motif interacts directly with the OB1 domain of POT1.

3.5. Heterodimerization of POT1 with TPP1 does not block recognition of the NT motif

In human cells, POT1 is normally present as part of a heterodimer with the TPP1 protein [43]. Importantly, this association of TPP1 with POT1 reduces the affinity of POT1 for RNA and increases the sequence specificity of the POT1/TPP1 dimer [44]. In this section, we examined the effects of TPP1 on the recognition of the NT motif by POT1. A human TPP1 protein purified from transfected human cells was purchased (OriGene Technologies Inc., Rockville, MD). Silver staining analysis of the purified protein revealed TPP1 as the main molecular species present (Fig. 6A). In titration experiments, increasing amounts of this purified TPP1 protein was added to a fixed concentration of POT1 in the presence of probe OB1/OB2 (Fig. 6C). As expected, the addition of the purified TPP1 protein led to the formation of a larger, slower migrating POT1/TPP1 complex (top arrowhead). In Fig. 6D, we compared the sequence specificity of this POT1/TPP1 dimer with that of the POT1 protein alone. No difference in sequence specificity was detected between the POT1 and POT1/TPP1 complexes (bottom and top arrowheads, respectively). For both complexes, the highest affinity probe was NT/OB1, followed by OB1/OB2, and then by the OB1 probe (NT/OB1 > OB1/OB2 ≫ OB1 ⋙ NT). Hence, the association of POT1 with TPP1 does not block recognition of the NT motif by POT1.

3.6. POT1 fails to recognize RNA molecules containing an NT/OB1 site

Lastly, we investigated whether human POT1 might bind to RNA molecules containing an NT/OB1 site. A single deoxythymidine in OB1/OB2 binding sites is what dictates the DNA versus RNA discrimination by the human POT1 protein [44]. This deoxythymidine is at the second position of the OB1 motif (underlined in 5′-TTAGGGTTAG-3′) and because this base is also part of the NT/OB1 site (underlined in 5′-TNCANNAGKKKTTAGGNT-3′), we predicted that the protein would fail to recognize an NT/OB1 site made of RNA. To test this hypothesis, we examined the binding of the purified human POT1 protein to an NT/OB1/OB2 probe made of either DNA (NT/OB1/OB2) or RNA (r(NT/OB1/OB2)) (Supplemental Fig. S2A). As predicted, POT1 formed a complex with the DNA probe, but was unable to recognize its RNA equivalent (Supplemental Fig. S2B).

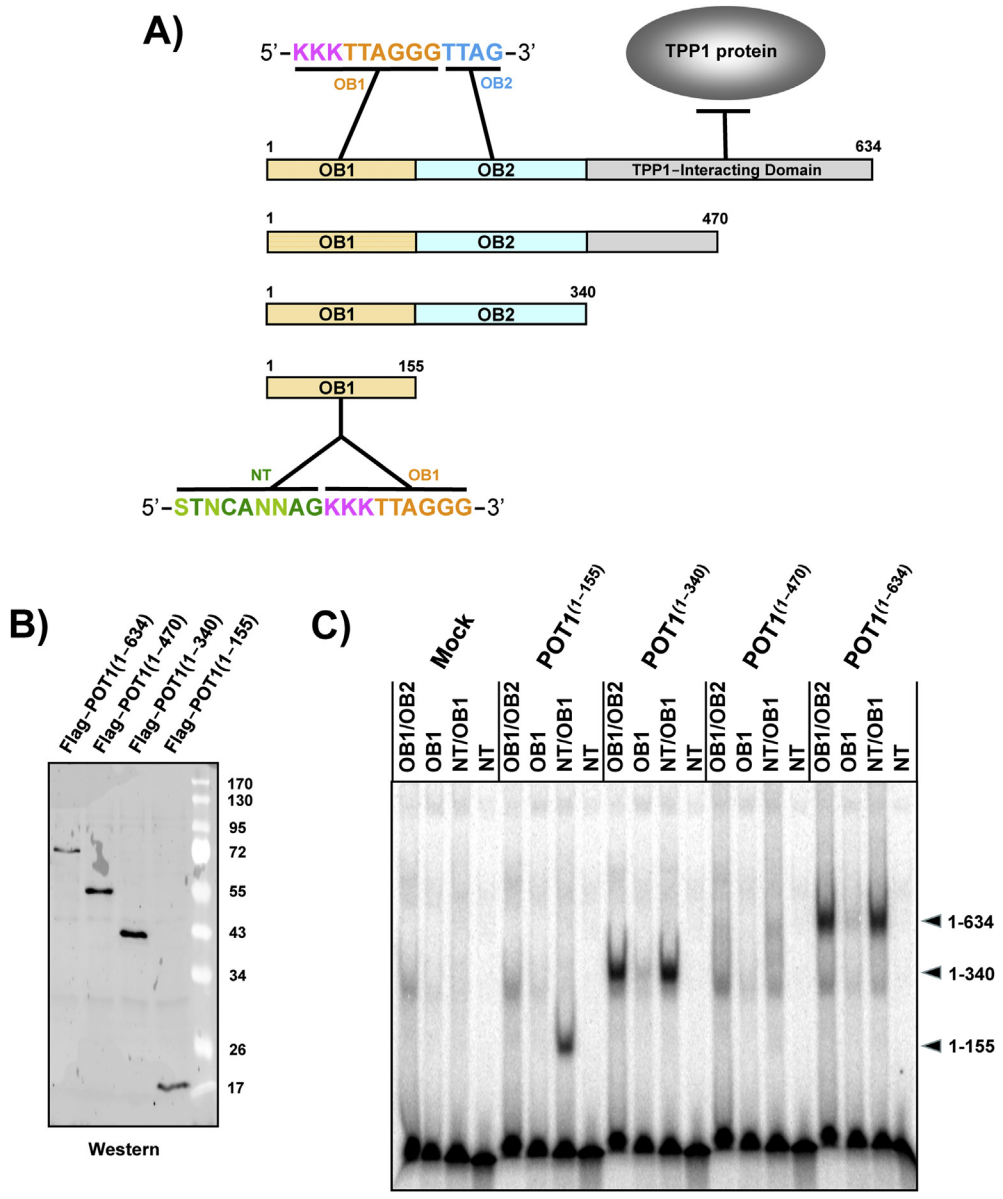


Fig. 4. The NT motif is recognized by the OB1 domain of POT1. A) Schematic description of the Flag-POT1 mutants tested and summary of the results. B) Western blot analysis of the Flag-POT1 mutants. The *in vitro* translated mutants were detected using the anti-Flag M2 antibody. C) EMSA with the *in vitro* translated Flag-POT1 mutants. Full length Flag-POT1 and its truncated mutants were incubated with the indicated radiolabeled probes (OB1/OB2, OB1, NT/OB1, and NT), after which protein/DNA complexes were resolved by electrophoresis. Migration of the Flag-POT1/DNA complex (arrowhead) was inversely proportional to the size the Flag-POT1 protein, with the Flag-POT1(1–155) protein migrating the fastest.

4. Discussion

Human POT1 binds telomeric DNA with high affinity and specificity [10], but the possibility that the protein might also recognize non-telomeric sites had not yet been formally investigated. Here, we have used SELEX to reexamine the sequence specificity of human POT1. Two classes of high affinity POT1 binding sites were retrieved by SELEX. The first class contained a single telomeric binding site for POT1 (5′-TTAGGGTTNG-3′). The second class carried a novel non-telomeric POT1 binding site that was made of both a telomeric (OB1 motif) and non-telomeric (NT motif) DNA element. We then showed that this NT motif is recognized by an additional DNA-binding surface located in the OB1 domain of POT1. This differs from the fission yeast POT1 protein, whose broad specificity is made possible by the flexibility of the Pot1pC OB-fold (OB2 equivalent) rather than by the presence of an additional DNA-binding surface (as in the human POT1 protein). The ability of human POT1 to bind preferentially to non-telomeric DNA binding sites raises the possibility that the protein may play a role at non-telomeric locations. In the next paragraph, we discuss the interactions of POT1 with each class of binding sites and the potential significance of the ability of POT1 to recognize non-telomeric DNA motifs.

4.1. Telomeric OB1/OB2 binding sites

Class I molecules had in common that they each carried a single telomeric DNA binding site for human POT1 (5′-TTAGGGTTNG-3′). In agreement with the literature, these sites were composed of juxtaposed OB1- and OB2-binding motifs. The orientation and

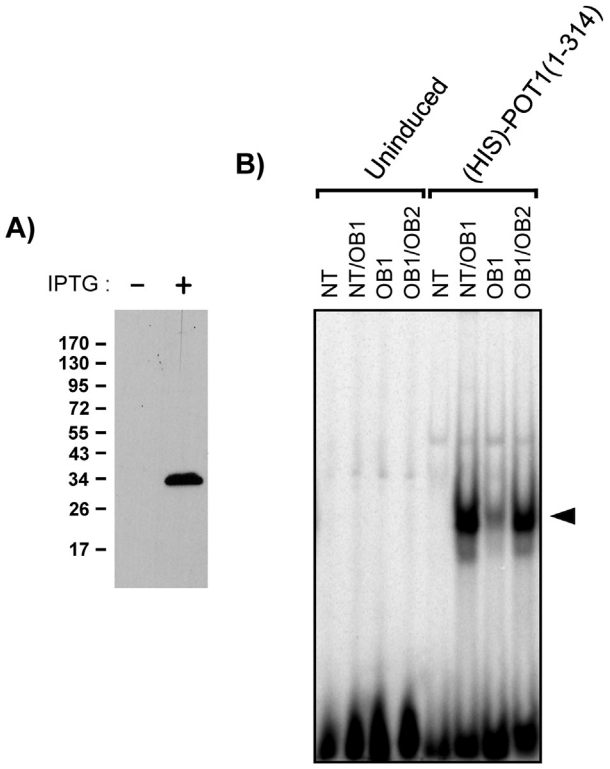


Fig. 5. The NT motif is recognized by His-tagged POT1(1–314) present in bacterial cell extracts. A) Western blot detection of the His-tagged protein after induction with IPTG. The protein was detected using a nickel (Ni^{2+})-activated derivative of horseradish peroxidase, HisProbe-HRP. Detection was following the manufacturer's instructions, except that blocking and binding were performed in the presence of 3 M guanidine-HCl. B) EMSA with extracts of *E. coli* cells induced or not for His-POT1(1–314) expression. Arrowhead: position of the His-POT1(1–314)/DNA complex.

spacing of the OB1 (5′-TTAGGG) and OB2 (TTNG-3′) motifs was as reported previously for human POT1 [10]. X-Ray crystallographic studies have revealed that the sequence specificity of the human POT1 protein is dictated by the number of hydrogen bonds that form between its OB1 and OB2 domains and the nitrogenous bases of the DNA [9,10]. The majority of these bonds are contributed by the OB1 domain (22/31 hydrogen bonds), with the remaining interactions contributed by the OB2 domain (9/31 hydrogen bonds). In accordance with the importance of the OB1 motif for POT1 binding, a perfectly well-conserved copy of the OB1 motif was present in all of the class I molecules. The OB2 motif was not as well-preserved but was still important for binding, since binding to class I molecules was inhibited by the deletion of the OB2 motif or by the deletion of the OB2 domain of POT1. The adenine at the third position of the OB2 motif (TTAG-3′) is the only base in telomeric binding sites that does not participate in hydrogen bonding interactions with POT1 [9,10]. Not surprisingly, this adenine was also the least well-conserved nucleotide of the class I consensus. Finally, an unexpected finding was that the OB1/OB2 sites in class I molecules were almost always flanked on both sides by a single thymidine (T/OB1/OB2/T). A thymidine at these two positions would not be expected to be present in telomeric DNA, so the significance of this finding is unclear. Overall, our class I data is consistent with the well-established DNA-binding properties and crystal structure of human POT1, including correlation between sequence specificity and number of hydrogen bonds involved as well as the requirements for the relative position and orientation of OB1 and OB2 motifs.

4.2. Non-telomeric NT/OB1 sites

Class II molecules had in common that they each carried a non-telomeric motif (5′-TNCANNAG) along with a single telomeric DNA motif (TTAGGN-3′). Follow-up studies indicated that the two motifs were recognized by the OB1 domain of human POT1, as binding was unaffected by the deletion of the OB2 domain and was still observed with the OB1 domain alone. Importantly, the recognition of class II sites by POT1 required the presence of both the telomeric (OB1) and non-telomeric (NT) motifs, since the omission of one motif or the other was sufficient to prevent binding. This requirement for the two motifs was observed with several different sources of human POT1 protein, including *in vitro* translated POT1, bacterially-produced POT1, and POT1 protein present in or purified from human cell extracts. This finding was also observed in the context of the POT1/TPP1 dimer, in spite of TPP1's known

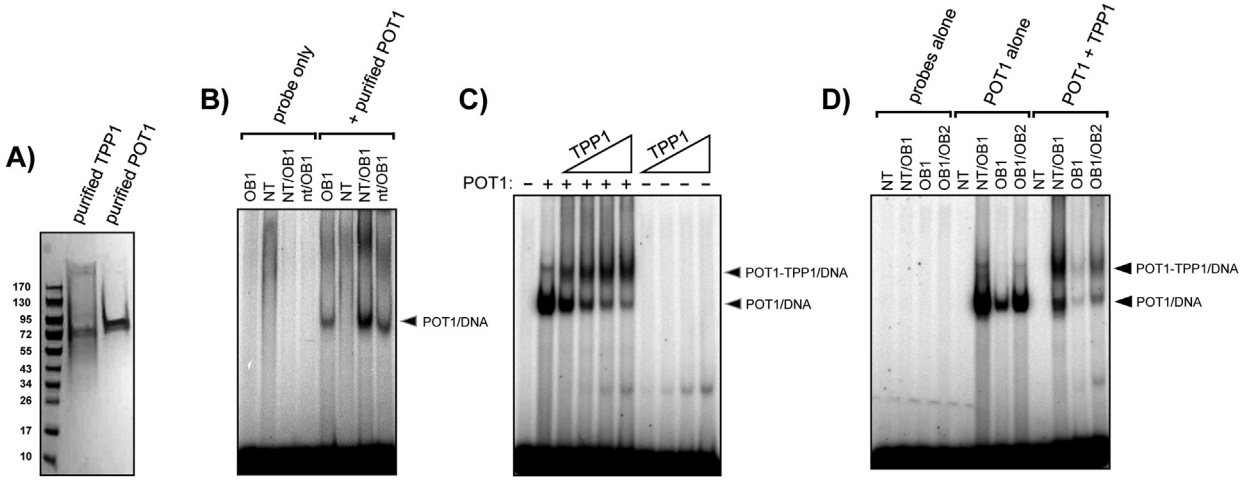


Fig. 6. The NT motif is recognized by purified POT1 and POT1/TPP1 dimer. A) Purity of the isolated POT1 (399 ng) and TPP1 proteins (163 ng), as determined by SDS-PAGE and silver staining. B) EMSA with the purified human POT1 protein. The purified protein (0.5 pmol) was incubated with the indicated radiolabeled probes and the complexes formed (arrowhead) were resolved by electrophoresis. C) Titration of the TPP1 protein for the formation of POT1/TPP1 dimers. EMSA was performed with (+) and without (–) POT1 (0.5 pmol) in the presence of an increasing molar excess of TPP1 (1×, 2×, 4×, 8×). Probe used was the canonical OB1/OB2 binding site. Arrowheads show the location of the POT1/DNA and POT1/TPP1/DNA complexes. D) Sequence specificity of the POT1/TPP1 dimer and its interaction with the NT motif. EMSA was performed with the indicated probes in the presence of either no protein, POT1 alone (0.5 pmol), or POT1 (0.5 pmol) plus TPP1 (2 pmol). Arrowheads show the location of the POT1/DNA and POT1/TPP1/DNA complexes.

propensity to restrict the DNA binding specificity of POT1 [44]. Thus, the NT motif is not merely an alternate ligand of the OB1 domain, but rather is an additional motif that the domain can recognize on top of the OB1 motif.

The OB1 and NT motifs synergize to increase the affinity of the human POT1 protein for DNA, thereby implying that the two motifs are recognized by distinct regions of the OB1 domain. Which amino acid residues of the OB1 domain are specifically involved in establishing contacts with the NT motif is unknown. The OB1 domain lacks sequence homology with other conserved domains known to bind ssDNA with sequence specificity, such as KH, RRM, and Whirly domains [45]. Instead, we propose that the binding surface that recognizes the NT motif is an extension of the DNA-binding grove of the OB-fold. In the 3D structure of human POT1 bound to 5′-TTAGGGTTAG-3′, the 5′-end of the DNA opens up to two valleys that could accommodate both the 3K and NT motifs (Blue arrows; Fig. 7). We have mutagenized some of these residues (N65A, E67A, I96A, S98G/S99G, S98A, S99A, A68D/S99A; yellow in Fig. 7), but we have not yet identified mutations that block NT motif recognition (Supplemental Fig. S3). Efforts are currently underway to co-crystalize POT1 with an NT/OB1 binding site to solve the 3D structure of the complex. Mutagenizing the amino acids involved in NT motif recognition might potentially identify POT1 mutants that could still locate to telomeres but fail to interact with NT motifs. Such mutants would represent an invaluable new tool to help elucidate the biological role of the NT-binding activity of POT1.

4.3. Significance of non-telomeric POT1 binding sites

If the POT1/TPP1 dimer has evolved to present a binding surface that recognizes the NT motif, then what is biological significance of this activity? Where in human cells would a POT1/TPP1 dimer find an NT/OB1 binding site? Human POT1 fails to bind to RNA molecules that contain an NT/OB1 site (Supplemental Fig. S2), and the quasi totality of the DNA in the genome is kept double-stranded, except for when chromatids are transcribed, replicated, or repaired. Yet, the great majority of ssDNA-binding proteins that function in these general processes bind DNA in a sequence independent manner (e.g. RPA, BRCA2, and PARP1). However, there is a multitude of proteins that bind ssDNA with sequence specificity, and most of these proteins play a role in either telomere maintenance and/or transcriptional regulation [45–47]. Many transcription factors have now been discovered that recognize specific ssDNA sequences. An example is the factors associated with the S1 nuclease hypersensitivity sites of the c-myc gene. These sites present with unusual secondary structures (G-quadruplex, I-motif) that serve as anchors for ssDNA-binding proteins that control the transcription of the c-myc gene, such as hnRNP K, FBP, and CNBP [45–47]. Similarly, POT1 could potentially control the transcription of genes that possess an accessible regulatory element harboring NT/OB1 and/or OB1/OB2 sites (Graphical abstract; Internal sites). Such dual roles in both telomere maintenance and transcriptional regulation have recently been ascribed to other telomere-associated DNA-binding proteins, including TRF1 and TRF2 [48,49]. In the promoter of genes, these POT1 binding sites could be accessible if they are part of local DNA structures (e.g. G-quadruplexes). The human POT1 has the ability to bind to OB1/OB2 sites embedded in a G-quadruplex, which the protein can then melt upon binding [48]. A search of the human genome revealed potential NT/OB1 binding sites within 10 kb of the transcriptional start site of a selected number of genes, within regions rich in transcriptional regulatory elements (Supplementary Table S1). Additional studies will be necessary to determine if these sites are occupied by POT1 (i.e. Chromatin immunoprecipitations) and if the protein regulate the expression of these genes (i.e. Expression studies).

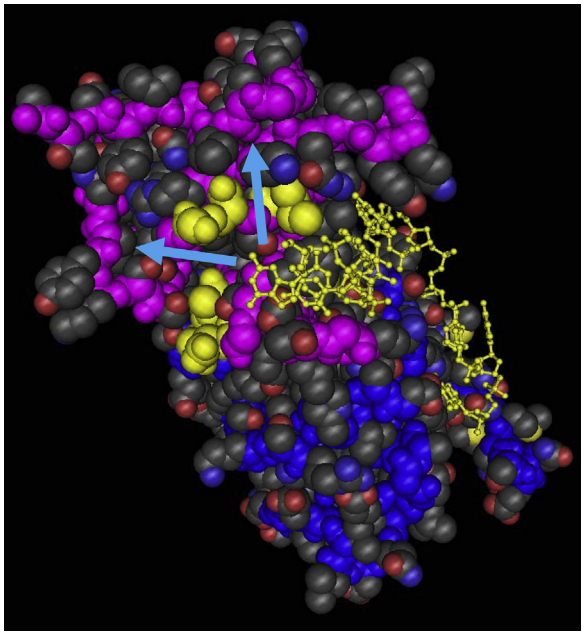


Fig. 7. Space fill model of the interaction of POT1 with an OB1/OB2 probe and location of mutagenized residues. The image shows the 3D structure of the human POT1 bound to 5′-TTAGGGTTAG-3′ (PDB: 3KJP). The OB1 backbone is displayed in purple color, the OB2 backbone is show in blue, and the DNA is yellow. The 5′-end of the DNA presents itself to two different paths that could accommodate an upstream NT motif (Light blue arrow). Amino acid residues along these paths were mutagenized (Yellow-painted residues). None of the mutants affected recognition of the NT motif (Supplementary Fig. S2).

4.4. Conclusion

The highest affinity target of human POT1 is a bipartite consensus made of both telomeric and non-telomeric DNA motifs. Recognition of these sites is achieved by means of the OB1 domain only and is unaffected by the dimerization of POT1 with TPP1. This affinity of human POT1 for non-telomeric binding sites raises the possibility that the protein may play additional roles at non-telomeric locations, in perhaps regulation of gene transcription. Future studies will be needed to elucidate the biological significance of this ability of POT1 to bind preferentially to non-telomeric sequences. Mutants of POT1 that can still bind telomeric DNA but lack the ability to recognize NT motifs will be instrumental in deciphering the role of POT1 at these non-telomeric locations.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2015.04.015.

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