HHS Public Access

Author manuscript

Biochemistry. Author manuscript; available in PMC 2017 June 13.

Published in final edited form as:

Biochemistry. 2017 March 28; 56(12): 1737–1745. doi:10.1021/acs.biochem.6b01236.

Characterization of DNA substrate binding to the phosphatase domain of the DNA repair enzyme, PNKP

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Abstract

Polynucleotide kinase/phosphatase (PNKP) is a DNA strand break repair enzyme that uses separate 5′ kinase and 3′ phosphatase active sites to convert damaged 5′-hydroxyl/3′-phosphate strand termini to ligatable 5′-phosphate/3′-hydroxyl ends. The phosphatase active site has received particular attention as a target for inhibition in cancer therapy development. The phosphatase domain dephosphorylates a range of single and double stranded substrates, however structural studies have shown that the phosphatase catalytic cleft can only bind single stranded substrates. Here we use a catalytically inactive but structurally intact phosphatase mutant to probe interactions between PNKP and a variety of single- and double-stranded DNA substrates using electrophoretic mobility shift assay (EMSA). This work indicates that the phosphatase domain binds 3′-phosphorylated single-stranded DNAs in a manner that is highly dependent on the presence of the 3′-phosphate. Double-stranded substrate binding, in contrast, is not as dependent on the 3′-phosphate. Experiments comparing blunt ended, 3′-overhanging, and frayed ended substrates indicate that the predicted loss of energy due to base pair disruption upon binding of the phosphatase active site is likely balanced by favorable interactions between the liberated complementary strand and PNKP. Comparison of the effects on substrate binding of mutations within the phosphatase active site cleft with mutations in surrounding positively charged surfaces suggests that the surrounding surfaces are important in binding to double-stranded substrates. We further show that while fluorescence polarization methods can detect specific binding of single-stranded DNAs with the phosphatase domain, this method does not detect specific interactions between the PNKP phosphatase and double-stranded substrates.

Introduction

The repair of breaks in the DNA phosphodiester backbone is critical for the maintenance of genomic stability in all organisms. DNA strand breaks are repaired by the combined actions of DNA polymerases and ligases that require 5′-phosphate and 3′-hydroxyl DNA termini for chain extension and ligation. DNA damage however often results in the generation of

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Supporting Information

DNA sequences, EMSA experiments

alternative chemical structures at DNA ends, which must be acted on by a host of DNA end-processing factors before repair can be completed[1]. 3′-phosphate and 5′-hydroxyl termini are common alternative DNA end structures that are generated by DNA damaging agents such as reactive oxygen species and ionizing radiation, as well as by the action of the DNA repair enzymes such as the NEIL DNA glycosylases and Tyrosyl DNA-phosphodiesterase 1 (TDP1). These 3′-phosphate and 5′-hydroxyl termini are specifically processed to 3′-hydroxyl/5′-phosphate ends by the DNA repair enzyme polynucleotide kinase/phosphatase (PNKP)[2].

The importance of PNKP for DNA repair in human cells was first demonstrated by the finding that stable down-regulation of PNKP expression leads to enhanced rates of spontaneous mutation and elevated sensitivities to a variety of DNA damaging agents[3]. PNKP mutations are associated with profound neurodevelopmental and neurodegenerative disorders[4–6], and PNKP deficiency in mouse models causes defects in DNA strand break repair leading to neurodevelopmental defects[7]. The notion that PNKP inhibition could sensitize tumour cells to DNA-targeting therapies[8, 9], and the finding that PNKP exhibits a synthetic lethal partnership with the tumour suppressors SHP-1 and PTEN[10,11] has driven recent efforts to identify PNKP small molecule inhibitors as potential anti-cancer agents. Preliminary inhibitor screening has identified promising small molecule PNKP inhibitors that sensitize human cells to DNA damage[12,13], and this effect is especially pronounced in cells deficient in SHP-1 or PTEN[10,11]

PNKP possesses a bifunctional catalytic domain containing separate 5′ DNA kinase and 3′ DNA phosphatase active sites, as well as an N-terminal FHA domain that interacts with distinct scaffolding proteins that facilitate the participation of PNKP in both single- and double-strand break repair pathways[14]. The kinase domain belongs to the adenylate kinase family and contains separate ATP and DNA substrate binding clefts. The phosphatase domain belongs to the haloacid dehalogenase (HAD) superfamily and contains a conserved catalytic motif Dx(D/T)x(T/V) in which the first Asp attacks the 3′-phosphate to form a covalent phospho-aspartate intermediate, while the second Asp/Thr acts as a general base in the hydrolysis of the phospho-aspartate enzyme-substrate intermediate. Biochemical studies have revealed that the kinase domain preferentially binds and phosphorylates 5′ termini within nicked, gapped or 5′-recessed substrates compared to either single-stranded or blunt-ended double stranded substrates[14–17]. Crystallographic studies reveal that double-stranded 5′ termini can bind to the kinase active site without base pair disruption[18] and the overall orientation of binding of substrate to the kinase domain in solution is supported by small angle X-ray scattering (SAXS) studies[16]. In contrast, the phosphatase domain has been shown to dephosphorylate a range of double- and single-stranded substrates with similar efficiencies, with the minimal substrate being a 3 nucleotide 3′-phosphorylated DNA[14, 17, 19] Crystallographic investigations have revealed that 3 nucleotide minimal substrates gain access to the narrow deep phosphatase active site through a mechanism that involves stacking of the two terminal unpaired substrate bases with phenylalanine residues in the active *site*[18,20] (Fig. 1). While there are no structures of PNKP bound to intact double-stranded phosphatase substrates, the packing of excess single stranded DNA strands against PNKP in one of the crystal structures has suggested a contiguous DNA binding surface

across both the phosphatase and kinase domains that may explain the binding of large double stranded DNA substrates[18].

The PNKP phosphatase domain has received particular attention as a potential inhibitor target and indeed the current lead PNKP inhibitors have been developed against this activity[12, 13]. However, our understanding of how diverse DNA substrates are recognized and processed by this domain is currently limited. Fluorescence experiments using 2-aminopurine-substituted DNA substrates have indicated that PNKP binding destabilizes base pairing in the terminal 2–3 base pairs of double stranded substrates[20]. To understand how the PNKP phosphatase domain interacts with double stranded substrates, we developed an electrophoretic mobility shift assay (EMSA) to directly assess the binding of WT PNKP and a series of mutants to diverse DNA phosphatase substrates. Our results support a model for PNKP-DNA interactions in which the enzyme uses its extensive positively charged kinase and phosphatase DNA binding surfaces to scan double stranded DNA for sites of flexibility containing strand break lesions. We suggest that base pair destabilization near the 3′-phosphate substrate is stabilized by electrostatic interactions between the partner strand and positively charged surfaces of the enzyme. A comparison of these results with results using a fluorescence polarization (FP) assay to monitor DNA binding reveals limitations of FP for the analysis of interactions with double stranded DNA substrates and suggests conditions for future FP-based high-throughput screens for novel inhibitors targeting the PNKP phosphatase domain.

Experimental Procedures

Cloning, Expression, Purification and Mutagenesis

Full-length mouse polynucleotide phosphatase/kinase (mPNKP) and its catalytic domain (residues 141-522; mPK) were cloned in pET-19b (Invitrogen) as a phosphatase-inactivated form (harbouring the D170A mutation). Clones were transformed to *E. coli* BL21 Gold cells, grown at 37 °C until od_{600} 0.6–0.8. Protein overexpression was induced by addition of 0.4 mM IPTG and growth at 18 °C for 16–20 hrs. The cells were harvested by centrifugation, and re-suspended in lysis buffer (150 mM KCl, 50 mM Tris HCl pH 8.3, 1 mM EDTA and 1 mM DTT) complemented with Halt™ protease inhibitor cocktail (ThermoFischer Scientific). Protein purification was performed according to the protocol previously described for human PNKP[21]. The purified proteins were stored in buffer containing 150 mM KCl, 10 mM Tris-HCl (pH 8.5) and 1 mM DTT at 4 °C. All mutants used in this manuscript were previously described[20].

Electrophoretic Mobility Shift Assay

Increasing concentrations of PNKP (0/0.02/0.04/0.08/0.2/0.4/0.8/2/4/8 μM) were titrated against a constant concentration (20 nM) of DNA substrate. In each reaction, 1 μL of DNA substrate, 1 μL of poly(dI-dC) (1 mg/mL), 1 μL of protein in storage buffer, 5 μL of EMSA binding buffer (150 mM KCl, 50 mM Tris-HCl (pH 7.5), 1 mM $MgSO_4$, 1 mM DTT and 10% glycerol) and 2 μL of H_2O were mixed and incubated for 10 minutes. 2 μL of 4X EMSA loading dye (40% glycerin, 240 mM Tris-HCl (pH 7.5) and 4 mg/mL bromophenol blue) was then added to the reaction mixture and loaded on a 6% pre-run native

polyacrylamide gel (19:1 acrylamide/bisacrylamide). Electrophoresis was carried out at 100 V for 55 min at 4 °C in 1X TBE buffer. Free and bound DNA substrates were visualized by Typhoon™ phosphorimager and analyzed qualitatively. See Supporting Information for the sequences of the DNAs used in these experiments.

Fluorescence Polarization Spectroscopy

Increasing concentrations of PNKP were titrated against a constant concentration of DNA substrate. 30 nM labeled DNA substrate was used in a reaction volume of 20 μL and dispensed in OptiPlate™ 384 F black microplates (PerkinElmer) after a 10 minute incubation. 6-carboxyfluorescein (6-FAM) fluorescence was excited at 480 nm and its emission was measured at 535 nm using an EnVision™ 2103 Multilable Plate Reader (PerkinElmer Inc.). The change in polarization was normalized and plotted against the logarithmic concentration of the protein. The polarization data were fit to a sigmoidal binding model by non-linear regression and the K_D was calculated for each DNA substrate using GraphPad Prism 6 (GraphPad Software, La Jolla, CA.).

Results

Characterization of PNKP binding to diverse DNA substrates by EMSA

To directly monitor binding of PNKP to a set of different DNAs representing the various substrates that would be processed by PNKP in either SSB or DSB repair processes, we developed an electrophoretic mobility shift assay (EMSA) using our $PNKP^{D170A}$ mutant (Fig. 2). Asp170 corresponds to the first aspartate of the conserved phosphatase motif Dx(D/T)x(T/V), and is proposed to attack the substrate 3′-phosphate[14, 17]. This mutant was previously demonstrated to specifically bind to DNAs containing 3′-phosphate groups with high affinity[17,20], and structural studies demonstrated that this mutation does not perturb PNKP structure or metal binding at the phosphatase active site[20]. As expected, $PNKP^{D170A}$ bound a 3′-phosphorylated 20-mer to form a complex with distinct mobility in EMSA. The stability of this complex was largely dependent on the 3′-phosphate as the same DNA sequence with a 3′-hydroxyl terminus bound much more weakly, forming complexes at much higher concentrations that migrated close to the well, suggesting that these complexes likely contained multiple PNKP protomers bound to a single DNA.

In the context of single strand break (SSB) repair and base excision repair (BER), PNKP is expected to process DNA termini within double stranded gaps and nicks, while in double strand break (DSB) repair, PNKP acts on blunt ended DSBs as well as DSBs with either 5′ or 3′ overhangs. We therefore also tested the binding of $PNKP^{D170A}$ to DNAs containing the same FAM-labeled 20-mer DNA annealed to different partner strands to yield a blunt-ended 20 base-pair duplex, a 20 base-pair duplex with a 16 nucleotide 5′ overhang, and a one nucleotide gapped DNA containing the same 20 base-pair duplex as well as a 15 base-pair duplex on the other side of the gap (Experimental Procedures). For each of these substrates, addition of low concentrations of PNKP resulted in formation of a complex that gave a tight band that was largely dependent on the presence of the 3′-phosphate termini, suggestive of binding of PNKP to the 3′-phosphate. However, as the PNKP concentration

was increased above ~0.4 μM, progressively lower mobility species were observed, suggesting binding of additional PNKP molecules.

The fact that these additional species were only observed in the DNAs containing double stranded substrates and not in the 20-mer single stranded DNA suggested that PNKP has an affinity for double stranded DNA. To test this, we repeated these experiments in the presence of excess unlabeled poly(dI-dC) competitor DNA (Fig. 2, + poly(dI-dC) panels). Indeed, addition of poly(dI-dC) competitor did eliminate the low mobility species in the titrations of the double stranded DNA substrates except at the highest protein concentrations. Interestingly, the first shifted complex formed was largely resistant to poly(dI-dC) competitor challenge, especially in samples containing a 3′-phosphate. These results confirm that the first complex represents a stable complex with the PNKP phosphatase active site engaged with the 3′ DNA terminus. Moreover, quantitation of the results in the presence of competitor suggest a hierarchy of binding affinity with gapped > overhang > blunt > single stranded DNA (Fig. S1).

The previous experiments were carried out with full-length PNKP containing both the catalytic and FHA domains. Previous work has suggested that XRCC1 or XRCC4 binding to the FHA can modulate PNKP catalysis[22–26], and that post-translational modification of the FHA-catalytic domain linker may also regulate PNKP function[27]. To directly test if the FHA or linker contribute to PNKP substrate binding, we repeated the EMSA experiments with the isolated PNKP catalytic domain (Fig. S2). The results show very similar patterns of substrate binding to full-length PNKP for the different DNA substrates. In these experiments, we also tested the binding of PNKP to a modified gap DNA substrate in which both the 3′ and 5′ ends of the gap are phosphorylated, to test the effect of the status of the 5′ end that could potentially engage the kinase active site. While we expected that the presence of the 5′-phosphate might inhibit binding, we did not see any evidence for a change in binding affinity comparing 5′-phosphorylated and non-phosphorylated substrates in the context of a 3′-phosphorylated gap. In the context of a 3′-hydroxyl- ended gap, the 5′-phosphorylated substrate actually appeared to have a slightly higher affinity for the DNA compared to the substrate with the 5′-hydroxyl terminus (Fig. S2).

Mutational analysis defines role of PNKP surfaces for substrate binding

Previous mutagenesis and crystallographic work illustrated a critical role for a set of residues within the phosphatase active site for both substrate binding and activity (Fig. 1)[18,20]. This work indicated that Phe305 and Phe184 both stack with the unpaired bases of the DNA, while Arg258 was also shown to be critical for binding the penultimate phosphate of the substrate. We tested the importance of these residues for DNA substrate interactions by EMSA (Fig. 3). As expected, mutation of any of these residues to alanine almost completely abrogates binding to the single stranded 3′-phosphate substrate. However, the impact of the mutations on binding of the double stranded substrates was less pronounced, especially for the gapped substrate. These results indicate that while these residues are critical for docking of the DNA within the phosphatase active site, they play a less important role in the docking of large double stranded DNAs to PNKP.

One potential region that has been postulated to play a role in the binding of the double stranded substrates is the positively charged 300–303 region (Fig. 1)[18,20]. We created a mutant in which these four positively charged residues were mutated to glutamic acid (300s). We predicted that if this region makes electrostatic contact with the double stranded portion of the substrate, then the charge swap mutant should exhibit greatly reduced binding for double stranded DNA. EMSA experiments show that this mutant, unlike R285A, F184A or F305A, is capable of binding 3′-phosphorylated single stranded substrates indicating that the mutation does not disrupt the phosphatase active site cleft. Strikingly however, this mutant shows no binding affinity for any of the double stranded substrates, suggesting that this surface is indeed critical for double stranded DNA recognition (Fig. 3).

We also tested the role of a positively charged region on α13 that lies between the phosphatase and kinase domains. We created a mutant in which Arg482 and Lys483 on α13 are mutated to glutamic acid (Fig. 1). This region was previously suggested to be involved in the kinking of nicked DNA substrates that interact with the PNKP catalytic domain and mutations of these residues to glutamic acid were previously shown to result in a subtle but significant reduction in kinase activity[18]. Consistent with the past results, this mutant was defective in binding both the single stranded as well as the double stranded DNAs, although its effect on the binding of the double stranded DNA substrates was not as pronounced as the 300s mutant (Fig. 4).

Electrostatics balance base-pair disruption in the binding of double stranded DNA substrates

The structure of the PNKP phosphatase bound to DNA suggested that ~3 base pairs at the 3′ terminus of double stranded DNA substrates must be disrupted to afford engagement of the 3′-phosphate terminus with the phosphatase active site. However, the energetic cost of the disruption of these base pairs would be expected to disfavor binding of double stranded DNA 3′ ends compared to single stranded DNA substrates. Yet, EMSA reveals that double stranded DNA substrates are actually bound more tightly than single stranded DNA substrates. To further probe the effect of base pairing at the 3′ end of phosphatase substrates, we compared the binding of two different DNAs based on the 20 bp double stranded DNA substrate. One DNA lacks the three 5′ nucleotides complementary to the 3′-phosphate end of the partner strand, resulting in a DNA with a three nucleotide, 3′ overhang. In the second DNA, the three 5′ nucleotides are non-complementary to the partner strand, resulting in a DNA substrate with non-complementary three nucleotide 5′ and 3′ overhangs. The EMSA results show that the 3′ overhang DNA binds PNKP slightly less well than the matched blunt ended 20 bp duplex DNA (Fig. 5). This result demonstrates that the pairing of the complementary strand to the substrate strand does not impair binding and in fact may actually facilitate binding. This result suggests that the complementary strand that is liberated through the engagement of the 3′ strand with the phosphatase active site must somehow facilitate DNA binding. The EMSA results with the 5′/3′ overhanging substrate support this hypothesis (Fig. 5). This DNA binds PNKP with a higher affinity than either the blunt or 3′ overhang substrate. We suggest that the enhanced binding of this substrate compared to the other two DNAs may be because in this case binding does not

require base pair disruption, and the 5′ overhang is free to engage with another surface of PNKP, and thereby provides additional binding energy.

Comparison with EMSA reveals limits of fluorescence polarization assay for monitoring PNKP-substrate interactions

We previously used fluorescence polarization spectroscopy (FP) to monitor PNKP-substrate interactions[20]. These results showed that the PNKP^{D170A} mutant could bind selectively to a 5 nt single stranded DNA substrate in a manner that was dependent on the presence of a 3′-phosphate terminus. Fluorescence polarization techniques provide a fast method to quantitate protein-nucleic acid interactions and the method can be modified to be used for high-throughput screening for small molecule inhibitors of these interactions[28]. The method requires that the interaction significantly reduce the tumbling rate of the complex compared to the labeled free substrate. To understand if FP could be used to monitor interactions between PNKP and larger DNA substrates, we carried out FP binding experiments with 5, 10, and 20 nt single stranded DNA substrates with and without a 3′-phosphate (Fig. 6A). The results of these experiments show that the three substrates with 3′-phosphate ends all bind PNKP with similar K_Ds of approximately 0.5–0.8 μM, similar to the concentration required for shifting of the 20 nt ssDNA by EMSA (Fig. 2). Removal of the 3′-phosphate dramatically reduced the binding affinity as measured by FP. The residual binding affinity of the substrates with the 3′-hydroxyl termini increased with the size of the substrate, and we suggest that this residual binding likely represents non-specific interactions between the DNAs and PNKP. Again, this is consistent with the EMSA results, which revealed non-specific interactions with the 20 nt 3′-hydroxyl substrate in the 2–8 μM range.

Given the correlation between the EMSA and FP results for the single stranded DNA substrates, we next tested binding of the double stranded DNA substrates to PNKP by FP (Fig. 6B). In this case, we obtained quite different results compared to EMSA. The FP binding affinities of the double-stranded blunt, overhang and gapped substrates were each significantly reduced compared to the single-stranded substrates. This is in marked contrast to EMSA, which indicated that the double stranded DNA substrates actually bind PNKP more tightly than the single stranded DNAs (Fig. 2, Fig. S1). The EMSA results suggested complex binding of PNKP to the double stranded DNA substrates, with an initial high affinity recognition of the 3′-phosphate by a single PNKP, followed by binding of additional PNKP molecules to other regions of the double stranded DNA substrate at higher concentrations. The apparent K_D for binding of PNKP to the double stranded DNAs measured by FP actually suggest that it may be only this latter non-specific interaction that is detected by FP, and not the high-affinity recognition of the 3′-phosphate by a single PNKP molecule. To test this, we performed parallel FP titrations in the presence of an excess of poly(dI-dC) as non-specific double stranded DNA competitor (Fig. 6B). We saw an ~2-fold decrease in the affinity for the double stranded DNA substrates, while we saw no decrease in the affinity for a single stranded DNA substrate containing a 3′-phosphate terminus. Taken together, these results suggest that FP is an efficient method to monitor specific binding of PNKP to DNA 3′-phosphate termini in single stranded DNA substrates but that FP, at least with our current experimental system, may not accurately report specific interactions between PNKP and 3′-phosphate termini in the context of double stranded

substrates. One explanation for the discrepancy might be that the enhanced rigidity of the double stranded DNAs compared to single stranded DNAs might reduce the sensitivity of detection for binding of a single PNKP to a double stranded substrate.

Discussion

Here we have developed a system to detect and characterize interactions between the PNKP phosphatase domain and diverse DNA substrates. Our results support a model where PNKP can non-specifically bind double-stranded DNA to scan for sites of strand break lesions, utilizing the extensive positively charged surfaces spanning the kinase and phosphatase active sites. Upon encounter of a strand break, the flexibility of the lesion could allow more extensive interrogation of the break, followed by limited melting of base pairs close to the 3′-phosphate terminus. We used the previous crystal structures[18,20] to develop a model of PNKP bound to a nicked, kinked DNA substrate that is consistent with the biochemical data (Fig. 7). The DNA in this model tracks across PNKP in a similar way to a previous model[17], however, our model differs in that it contains a nicked DNA in which both phosphatase and kinase active sites are engaged (Fig. 7). This model is also consistent with previous results that showed that inactivation of the phosphatase domain can inhibit kinase activity[17] since an inactive phosphatase domain would stably bind substrate and block product turnover. Our results suggest the energetic cost associated with the disruption of base-pairing is countered through favorable interactions between the liberated 3′-strand and the phosphatase active site, as well as electrostatic interactions between the complementary strand and positively charged surfaces.

The dramatic enhancement of binding affinity of PNKP for single-stranded 3′-phosphorylated DNAs compared to 3′-hydroxyl DNA demonstrates the importance of the 3′-phosphate for substrate recognition. Dephosphorylation of the substrate in the context of the wild type enzyme would be expected to drive the product strand from the active site, however, the more extensive interactions between PNKP and large double stranded substrates stabilize interactions with the 3′-hydroxyl product. Insight into product interactions have come from a crystal structure of the wild type PNKP bound to a 3′-hydroxyl DNA (PDB code: 3ZVM)[18]. In this structure, the 3′-hydroxyl strand remains bound to the protein, however the 3′-end is shifted out of the active site and into a groove leading away from the active site and bounded by the β13-α6 loop. This movement appears to be accommodated with only subtle conformational changes in PNKP, the most notable being a rotation of the Phe305 side chain to maintain stacking interactions with the 3′ DNA base.

Our results indicate a critical role for the positively charged β13-α6 loop (residues 300-303) for recognition of double-stranded phosphatase substrates. Charge reversal mutations in this loop do not impact binding to single stranded substrates but block binding of double stranded substrates (Fig. 4) and our model of the interaction of PNKP with a double stranded DNA is consistent with the proximity of this loop to the DNA (Fig. 7D). Interestingly, this loop is absent in the T4 phage PNK phosphatase domain (Fig. 7E). This loop also supplies Phe305, which is critical for stacking with the 3′-terminal base. In the T4 enzyme, this residue is missing, however, its role might be filled by T4 Phe301, which spatially aligns

with Phe305 of murine PNKP. The other loop critical for stacking interactions with the substrate strand is the β10-α1 loop, which contains Phe184 that stacks primarily with the penultimate base. This loop is conserved in the T4 enzyme and contains Tyr179, which appears to be homologous to Phe184 in murine PNKP. Thus, while the T4 phosphatase active site cleft appears to contain critical aromatic residues for stacking with single stranded substrates similar to mPNKP, the lack of a key surface for the binding of double stranded substrates may suggest that the T4 enzyme recognizes its targets, such as a nicked tRNA anticodon loop[29], via a different mechanism.

The finding that PNKP exhibits a synthetic lethal relationship with the human tumour suppressors PTEN[11] and SHP-1[10], together with the finding that PNKP depletion sensitizes tumour cells to DNA damaging agents[3] has inspired efforts to uncover PNKP inhibitors as leads for cancer therapy development. Limited screening of a small library of polysubstituted piperidines has uncovered promising small molecule inhibitors with dissociation constants in the range of ~400 nM that are able to inhibit both the kinase and phosphatase activities of the enzyme and also sensitize cells to DNA damage in a PNKP-dependent manner[12,13]. The PNKP phosphatase active site cleft provides the most extensive pocket for the potential binding of inhibitors within the catalytic domain. However the inhibitors uncovered to date likely do not act by binding this cleft as they do not competitively block DNA substrate binding and appear to instead act in an allosteric manner that leads to a conformational change in the protein structure[13]. Fluorescence polarization using small ssDNAs targeted to the phosphatase active site could provide an excellent assay to uncover new classes of competitive PNKP inhibitors for cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank A. MacMillan for helpful comments on the manuscript.

Funding sources

This work was funded by NCI program project grant PO1CA092584 to JNMG, CIHR grant 114975 to JNMG and MW, CIHR grant MOP15385 to MW, and NSERC Discovery grant 2016-05163 to JNMG.

Abbreviations

PNKP	polynucleotide kinase/phosphatase
EMSA	electrophoretic mobility shift assay
FP	fluorescence polarization
HAD	haloacid dehydrogenase
SAXS	small angle X-ray scattering
6-FAM	6-carboxyfluorescein

SSB	single strand break
DSB	double strand break
BER	base excision repair
poly(dI-dC)	poly(deoxyinosine-deoxycytosine)
300s	PNKP quadruple mutant - R300E,K301E,K302E,K303E

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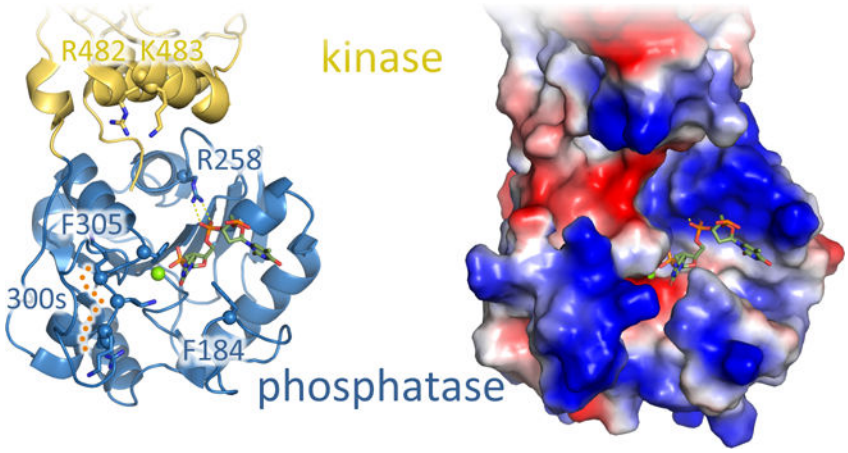

Figure 1.

Structural environment of the PNKP phosphatase active site. The view is into the PNKP phosphatase active site cleft with a bound single stranded 3′-phosphate DNA. In the left panel, residues critical for single strand DNA binding, F305, F184 and R258 are labeled, as are residues predicted to contact double stranded substrates (R482, K483, and residues within the 300s cluster – R300, K301, K302 and K303). The left panel shows an electrostatic surface representation of the same view. Figure was prepared in Pymol v1.7.6.4 (www.pymol.org).

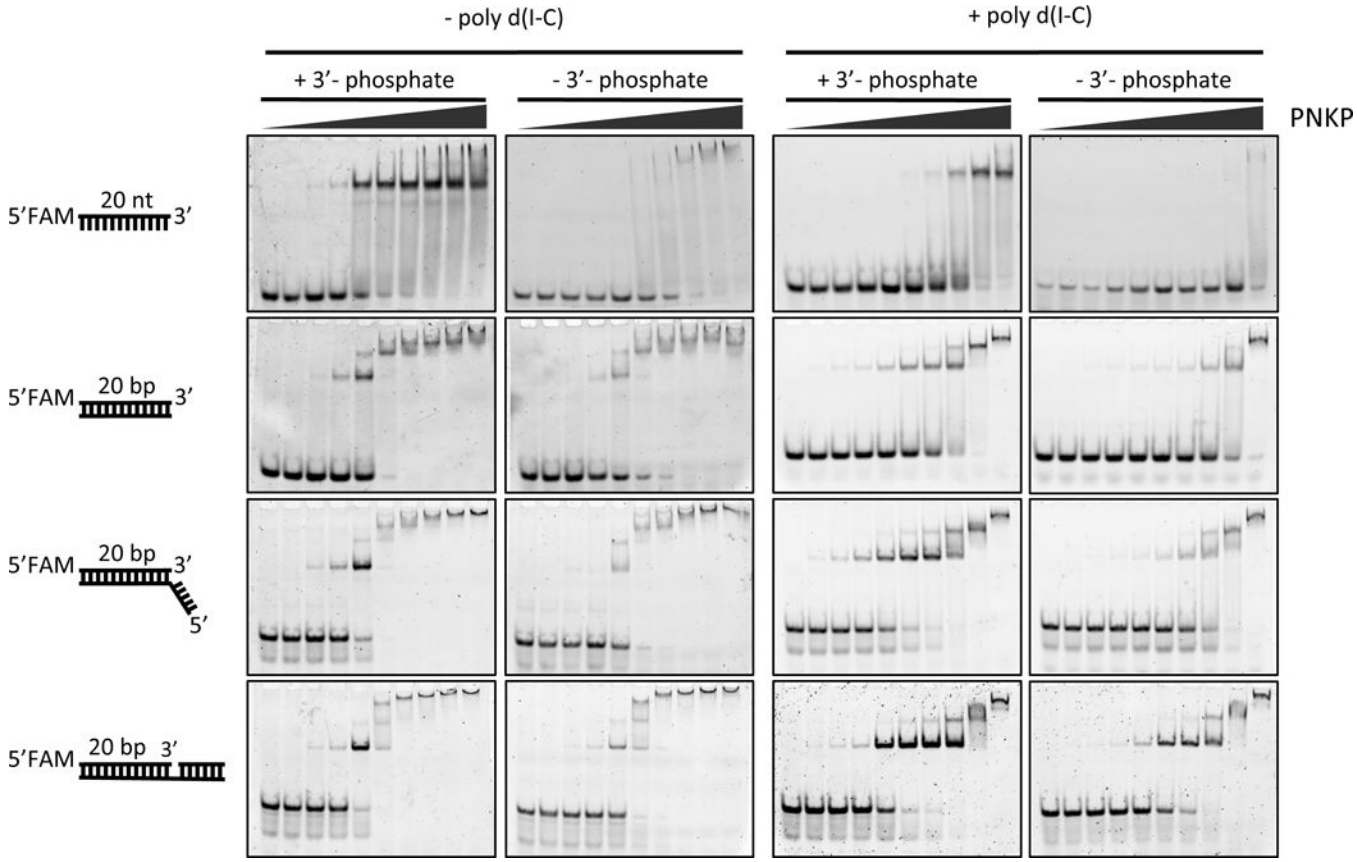

Figure 2.

Analysis of PNKP binding to diverse DNA substrates by EMSA. In each panel, increasing concentrations of PNKP (0/0.02/0.04/0.08/0.2/0.4/0.8/2/4/8 μM) were titrated against 20 nM of the DNA indicated on the left. The experiments were performed with or without 1 μg poly (dI-dC) competitor as indicated. Experiments were also performed with DNAs containing a 3′-phosphate or 3′-hydroxyl terminus on the 5′FAM-labeled as indicated.

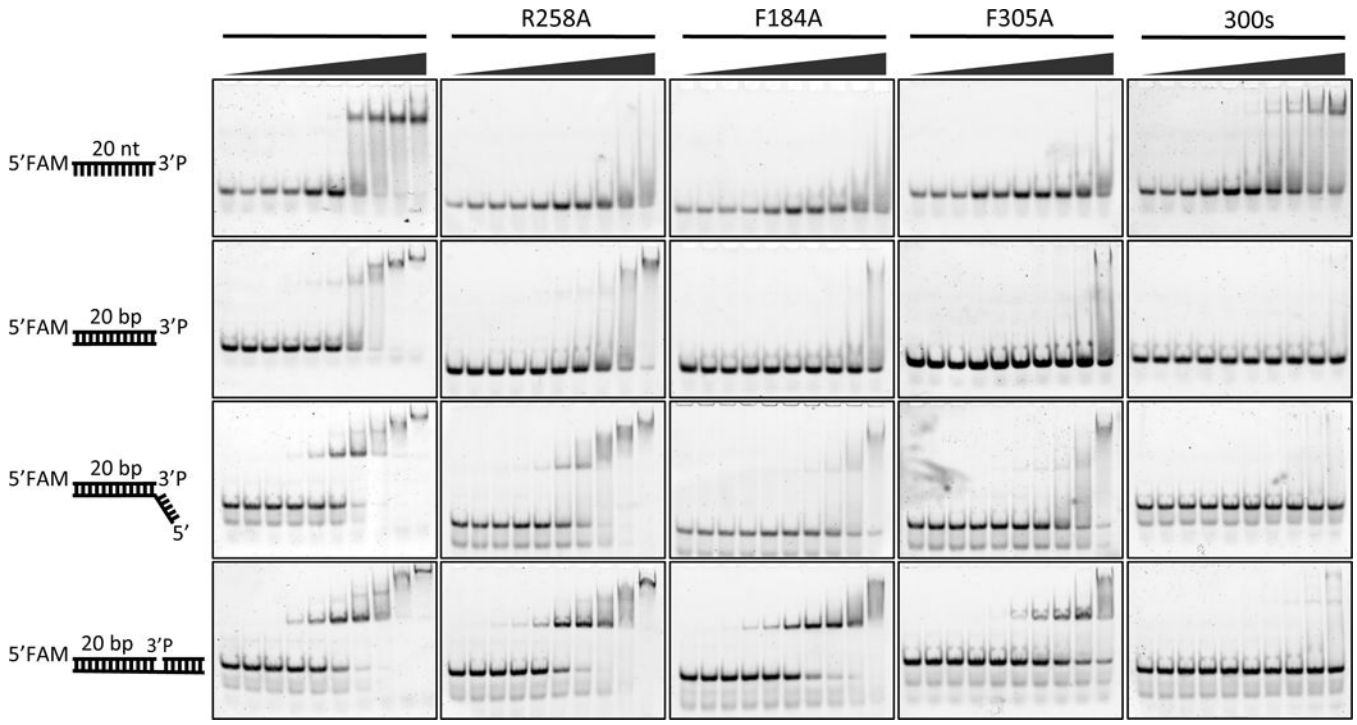

Figure 3.

Effects of phosphatase domain mutations on DNA substrate binding. The purified PNKP catalytic domain proteins indicated were titrated against the indicated DNA substrates as in Fig. 2.

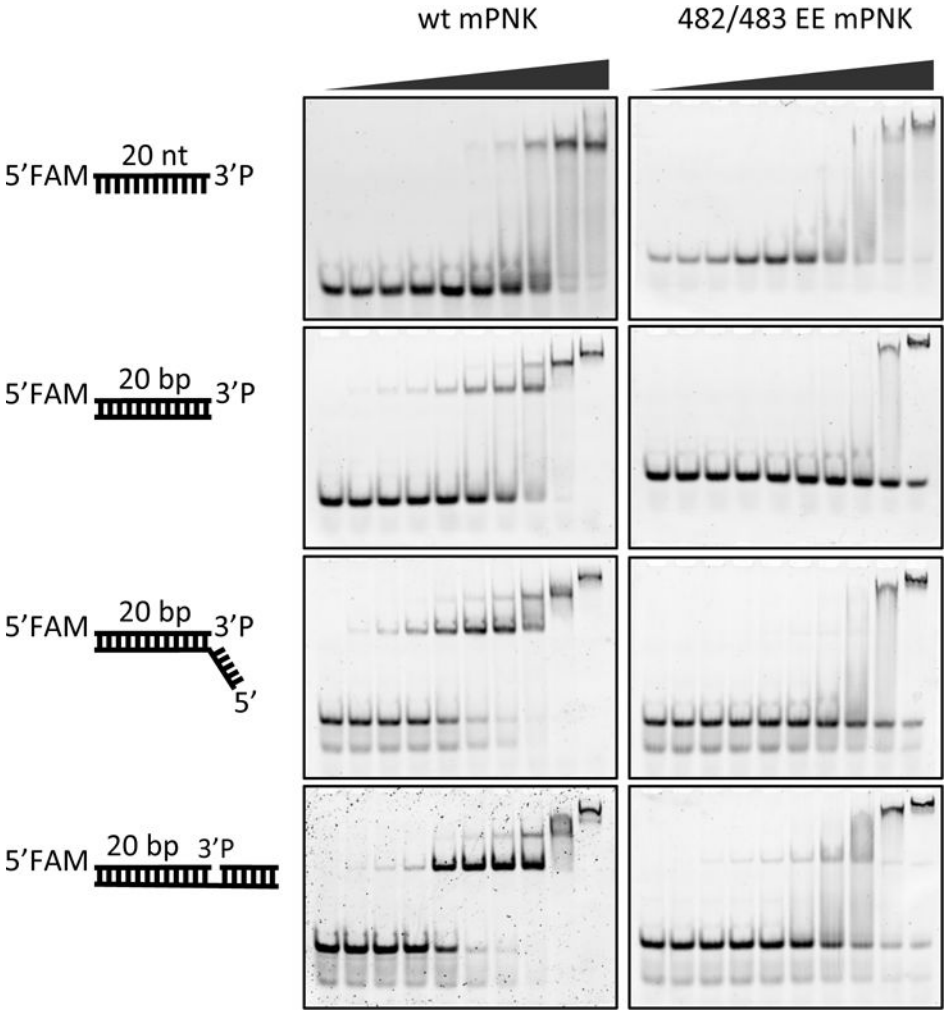

Figure 4.

Effects of mutations in α13 on DNA substrate binding. Experiments were performed as in Fig. 2 using full length PNKP (FHA + catalytic domain) or full length protein containing the indicated mutations.

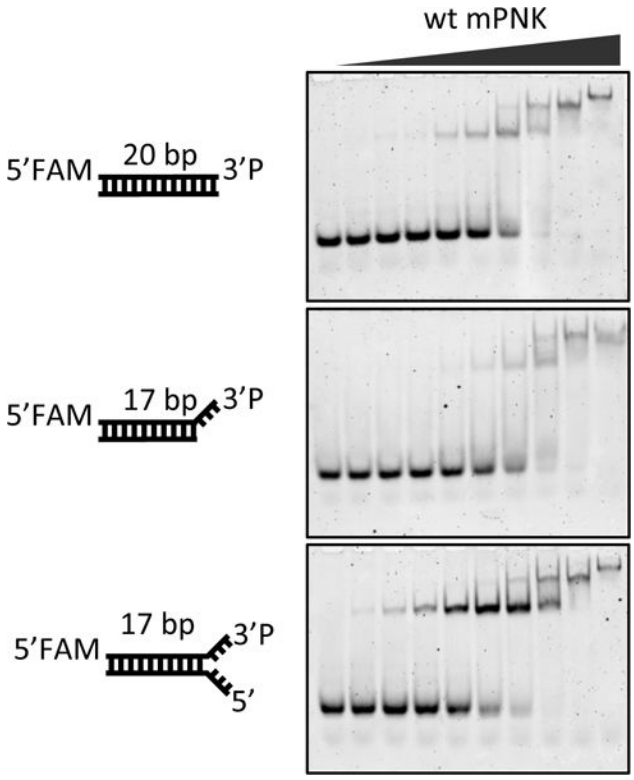

Figure 5.

Effect of base pairing on PNKP phosphatase substrate recognition. PNKP catalytic domain was titrated against the indicated DNA substrates as described in Fig. 2.

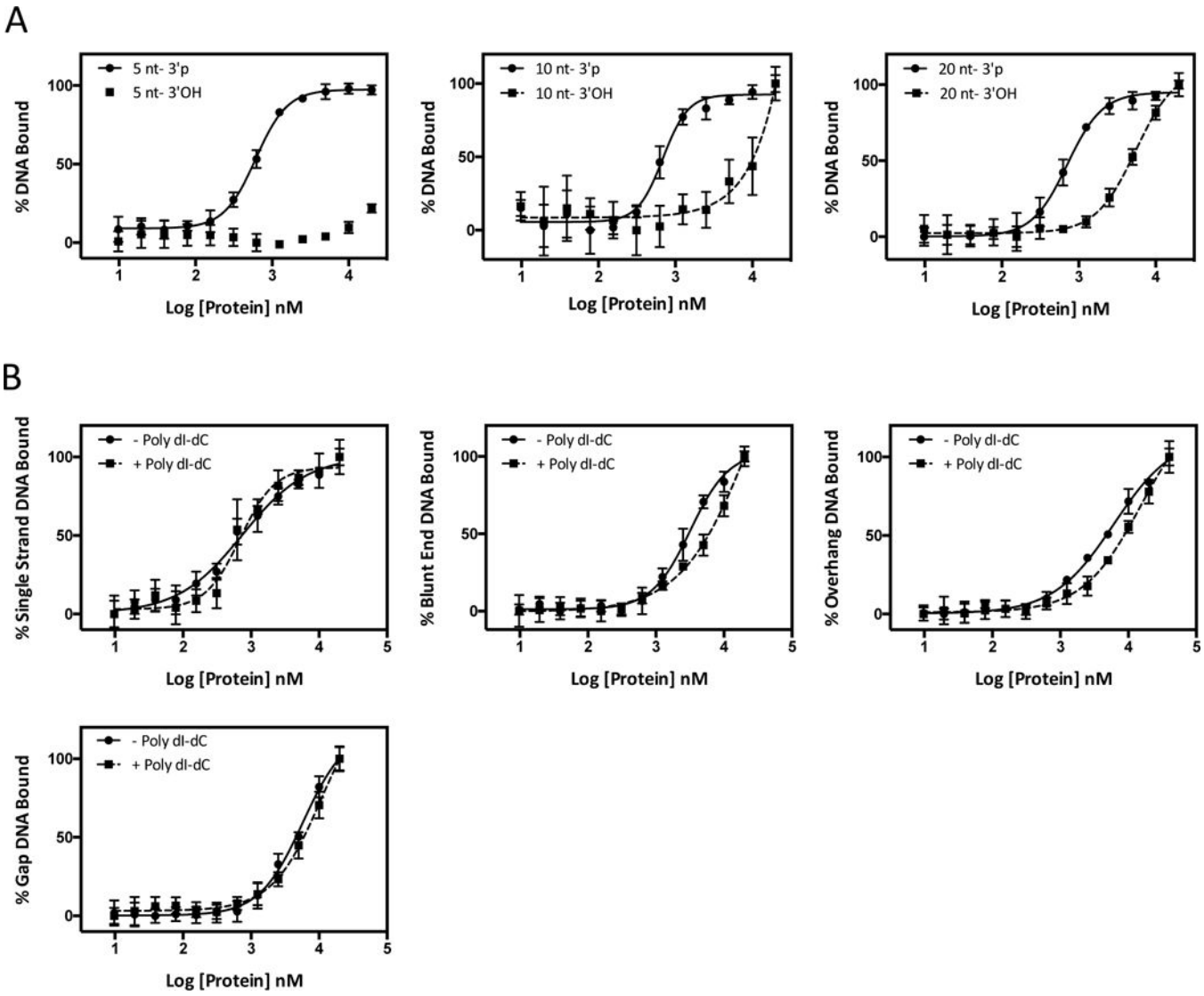

Figure 6.

Analysis of PNKP binding to single-stranded and double-stranded DNA substrates by fluorescence polarization (FP). (A) Comparison of the binding of different length single stranded DNA substrates to PNKP. Increasing concentrations of PNKP catalytic domain was titrated against the indicated single stranded DNA substrate and the data was fit to a single site binding model. Each titration point was determined in triplicate and each titration was replicated at least 3 times. Dissociation constants (K_Ds) determined for the 5 nt, 10 nt, and 20 nt 3′-phosphorylated were 0.53±0.11, 0.66±0.04, and 0.76±0.07 μM, respectively. K_Ds for the 3′-hydroxyl DNAs could not be accurately determined because these titrations did not achieve saturation. (B) Comparison of the binding of different double stranded DNA and single stranded DNA substrates to PNKP. Titrations against the indicated double stranded DNA substrates were carried out as in (A), except the titrations were carried out in the presence or absence of 1 μg poly(dI-dC) competitor DNA.

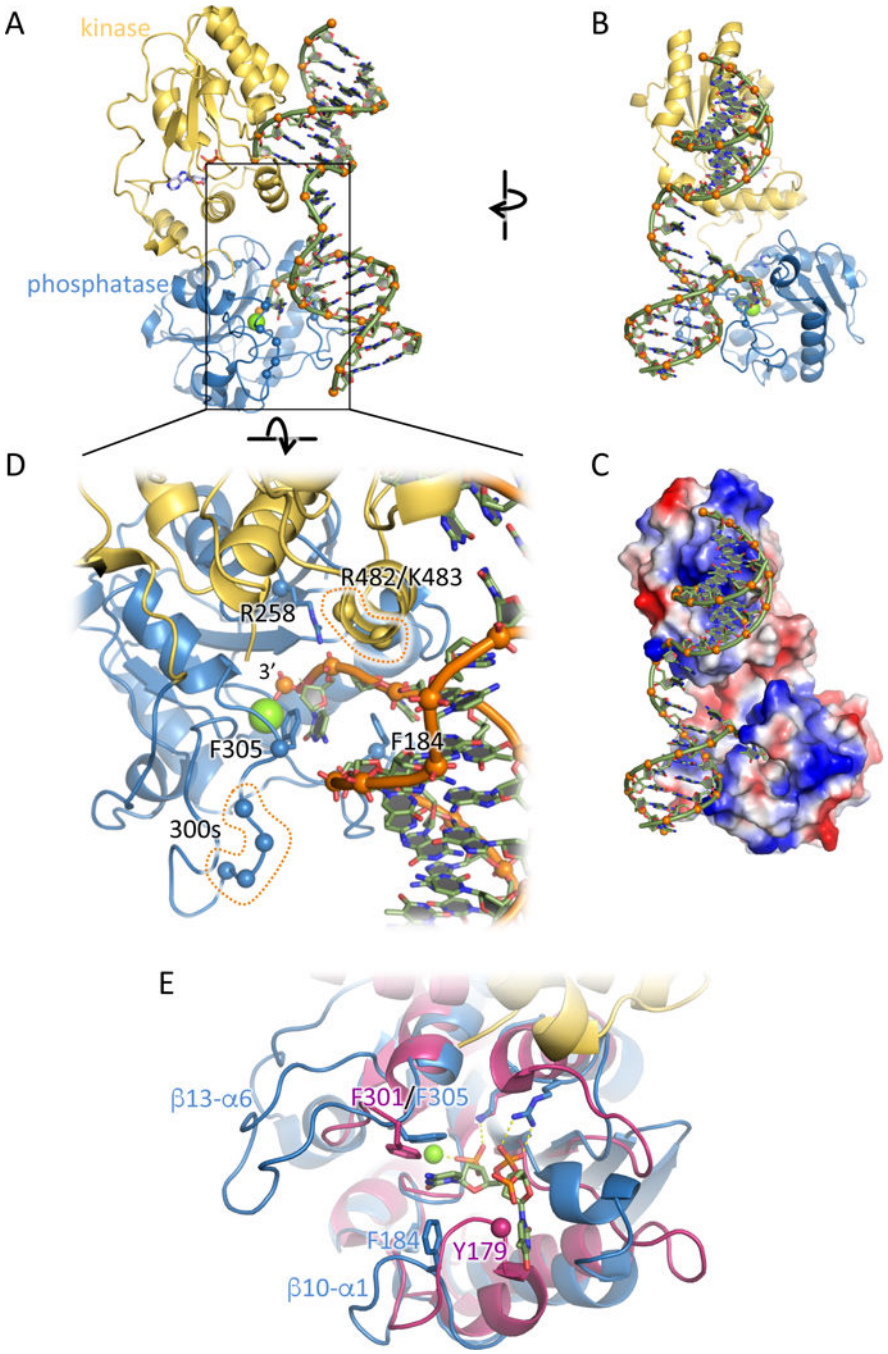

Figure 7.

A model for PNKP binding to a nicked DNA substrate. (A) Overview of the model with the PNKP kinase domain in yellow, PNKP phosphatase in blue, ADP in light blue, catalytic magnesium ion in light green, and DNA in green. (B) View of the model rotated ~90° about a vertical axis compared to the view in (A). (C) View of the model in the same orientation as (B) with an electrostatic surface displayed for PNKP. (D) Detail of the PNKP phosphatase active site. Key side chains for single stranded DNA substrate recognition are displayed as sticks, and additional residues that make additional electrostatic interactions with the DNA

are indicated with Cα backbone spheres. 300s refers to the positively charged amino acids 300–303. (E) Overlay of mammalian PNKP bound to DNA (blue and yellow; DNA in green, PDB code: 3U7F) with the phosphatase domain of T4 phage PNK (magenta, PDB code: 1LTQ), created by structural alignment of conserved residues in the phosphatase active site. Residues involved in stacking interactions with the DNA substrate are labeled (note that the side chain for T4 phage Y179 is not shown as this residue was not modeled in 1LTQ). Figures were prepared in Pymol v1.7.6.4 (www.pymol.org).