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The phosphatase activity of mammalian polynucleotide kinase takes precedence over its kinase activity in repair of single strand breaks

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ABSTRACT

The dual function mammalian DNA repair enzyme, polynucleotide kinase (PNK), facilitates strand break repair through catalysis of 5′-hydroxyl phosphorylation and 3′-phosphate dephosphorylation. We have examined the relative activities of the kinase and phosphatase functions of PNK using a novel assay, which allows the simultaneous characterization of both activities in processing nicks and gaps containing both 3′-phosphate and 5′-hydroxyl. Under multiple turnover conditions the phosphatase activity of the purified enzyme is significantly more active than its kinase activity. Consistent with this result, phosphorylation of the 5′-hydroxyl is rate limiting in cell extract mediated-repair of a nicked substrate. On characterizing the effects of individually mutating the two active sites of PNK we find that while site-directed mutagenesis of the kinase domain of PNK does not affect its phosphatase activity, disruption of the phosphatase domain also abrogates kinase function. This loss of kinase function requires the presence of a 3′-phosphate, but it need not be present in the same strand break as the 5′-hydroxyl. PNK preferentially binds 3′-phosphorylated substrates and DNA binding to the phosphatase domain blocks further DNA binding by the kinase domain.

INTRODUCTION

Damage to DNA is a causative factor in aging and a number of human disease processes including cancer. Single strand breaks (SSB) are a common type of damage to DNA, which can arise through both direct scission of the DNA backbone or as intermediates in normal DNA metabolic processes, such as replication and repair. Since strand breaks are both recombinogenic and cytotoxic, it is essential that they are repaired promptly and efficiently. However for strand resynthesis and ligation to proceed, the DNA termini must consist of a 3′-hydroxyl and a 5′-phosphate. In practice, SSB often have alternative termini, which must be processed before repair can be completed. 3′-Phosphate termini are common and can arise from a number of sources including reactive oxygen species, ionising radiation (1), Tdp1 processing of stalled topoisomerase I complexes, such as are generated by the drug camptothecin (2), and as intermediates in a sub-pathway of base excision repair of oxidative base damage (3). 5′-Hydroxyl termini are also commonly occurring products of strand scission by e.g. ionising radiation (4) or camptothecin treatment (5).

First identified in the mid 1970s (6), mammalian polynucleotide kinases (PNK) are bifunctional enzymes with both 5′-kinase and 3′-phosphatase activities (7,8). Human PNK is a 57 kDa protein (9,10), which has been implicated in both SSB repair and double strand break (DSB) repair via the non-homologous end joining (NHEJ) pathway (11–14). PNK forms multi-enzyme complexes *in vivo* with Pol β, XRCC1 and DNA Ligase III (14) and Tdp1, XRCC1 and Ligase III (15). Each of these complexes is necessary and sufficient to carry out particular sub-pathways of SSB repair. Stable down-regulation of PNK in human cells results in hypersensitivity to a number of DNA damaging agents including ionising radiation, camptothecin and H_2O_2 and also increases the spontaneous mutation frequency (16). It is therefore likely that PNK is a key player in protecting against both exogenous and endogenous sources of DNA damage.

Mammalian PNK enzyme is monomeric in structure (17) with the kinase and phosphatase domains being tightly associated and inseperable by proteolysis, while still showing some flexibility in orientation (18). The recent crystal structure of mouse PNK revealed that the two active sites are situated on the same side of the protein; however their physical separation (∼40 Å) seemingly precludes the possibility that the enzyme is able to simultaneously process 3′-phosphate and 5′-hydroxyl termini when they are located in the same nick or small gap (18). A key question is therefore how the dual activities of PNK co-ordinate with each other. To address this, we have

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used a novel system with a fluorescently double-labelled substrate, examining how the kinase and phosphatase activities compare in processing SSB flanked by both a 3′-phosphate and 5′-hydroxyl. We have also carried out individual site-directed mutagenesis of the kinase and phosphatase active sites of human PNK and show that while disruption of the kinase domain leaves the phosphatase activity unaffected, mutation of the phosphatase domain also abrogates the kinase activity on model nicked and gapped substrates containing a 3′-phosphate. To put our observations into a biological context we have also characterized the repair of a model substrate by human cell extracts.

MATERIALS AND METHODS

Materials

Synthetic oligonucleotides and chemical reagents were obtained from TAGN and Sigma–Aldrich, respectively, unless stated otherwise. MES was pre-treated with Dowex 1 × 2 chloride form before use, as preliminary experiments showed inhibition of hPNK by the untreated buffer, presumably by contaminating oligo(vinylsulfonic acid) as has been observed for RNase A (19).

Cloning, expression and purification of hPNK and mutants

The bacterial expression vector pETPNK was constructed by cloning the coding sequence of human PNK, amplified from vector kindly provided by Dr M. Weinfeld, into the NdeI and BamHI sites of pET28a (Novagen), with the appropriate restriction sites being introduced at either end of the coding sequence by PCR. Site-directed mutagenesis of the pETPNK plasmid to obtain the K378A, D171A and D173A mutants was carried out by the QuikChange (Stratagene) method. N-terminally His-tagged wild-type and mutated hPNK were expressed in *Escherichia coli* Rosetta 2 (DE3) pLysS cells (Novagen) induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. Purification of proteins was carried out over a Ni-NTA agarose (Qiagen) column according to the manufacturer's directions. Purified proteins were subsequently dialysed into buffer containing 50 mM HEPES (pH 7.8), 100 mM KCl, 1 mM EDTA, 1 mM DTT and 10% glycerol. Protein concentration was measured by ultraviolet (UV) absorbance at 280 nm in 6 M guanidine using a calculated extinction coefficient for the His-tagged protein of 62 730 $M^{-1}cm^{-1}$.

Preparation of substrate

Fluorescently labelled oligonucleotides and the unlabelled complementary strands were purchased from Eurogentec and purified by denaturing PAGE in a 20% gel. The oligonucleotides were annealed in 10 mM Tris (pH 7.5), 200 mM NaCl and 1 mM EDTA before purification in an 8% non-denaturing polyacrylamide gel. The purified oligonucleotide duplexes were ethanol precipitated and then redissolved at a concentration of 500 nM in 10 mM Tris (pH 7.5), 200 mM NaCl, 1 mM EDTA.

Activity assays

Double-stranded oligonucleotide substrates (50 nM) were incubated with the indicated amounts of wild-type or mutated hPNK for 10 min at 37°C in buffer containing 50 mM MES (pH 6.0), 20 mM NaCl, 10 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM EDTA and 1 mM DTT. Reactions were quenched by addition of an equal volume of formamide loading buffer (10 mM EDTA, 98% formamide, 10 mg/ml blue dextran) and heated to 95°C for 3 min before loading on a 20% denaturing polyacrylamide gel. Gels were visualized by fluorimagery using a Typhoon 9410 laser scanning system (Amersham). All experiments were repeated at least three times and representative gels are shown.

Competition experiments

Competitor oligonucleotide duplex was added at the indicated concentrations to reaction mixtures containing a fluorescently-labelled double-stranded oligonucleotide with a recessed 5′-hydroxyl in buffer containing 50 mM MES (pH 6.0), 20 mM NaCl, 10 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM EDTA and 1 mM DTT. Reactions were initiated by addition of PNK D171A mutant to a final concentration of 4 nM. Following incubation at 37°C for 8 min, the reactions were quenched by addition of an equal volume of formamide loading buffer (10 mM EDTA, 98% formamide, 10 mg/ml blue dextran) and heated to 95°C for 3 min before loading on a 20% denaturing polyacrylamide gel. Gels were visualized by fluorimagery using a Typhoon 9410 laser scanning system (Amersham). All experiments were repeated at least three times and a representative gel is shown. Band intensities were measured using ImageQuant software and the amount of product generated in each case was calculated as the intensity of the product band as a proportion of the total intensities of the product and substrate bands. Curve fitting was carried out by non-linear regression using SigmaPlot. The K_i is calculated as the distance between the X-intercepts of the linear plots (20).

Nitrocellulose filter-binding assays

Nitrocellulose filter-binding assays were carried out in a Bio-Dot SF slot blot apparatus using Hybond-ECL membrane (GE Healthcare) pre-treated with 0.4 M KOH for 10 min, washed and then equilibrated in binding buffer [50 mM MES (pH 6.0), 20 mM NaCl, 10 mM $MgCl_2$, 0.1 mM ATP, 1 mM DTT] for 1 h. Binding reactions containing binding buffer, 100 pM FAM-labelled substrate and the indicated amounts of enzyme were incubated on ice for 20 min before application to the membrane. Slots were washed with 200 µl of binding buffer and the membrane was then visualized by fluorimagery using a Typhoon 9410 laser scanning system. Binding curves were plotted as the proportion of DNA bound (corrected for non-specific binding to the filter) versus the log of the protein concentration and fitted by non-linear regression using SigmaPlot. The K_d was calculated as the concentration of enzyme which gave half maximal binding.

Repair by whole cell extracts

HeLa S3 cells were grown in adherent culture at 37°C and 5% CO_2 in MEM medium (Invitrogen) supplemented with 10% FCS (Cambrex), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all Invitrogen). Whole cell extracts were prepared from actively-dividing cells according to the method of Tanaka (21) as modified by Vodenicharov (22) and dialysed overnight against buffer containing 25 mM HEPES–KOH (pH 7.9), 2 mM DTT, 12 mM $MgCl_2$, 0.1 mM EDTA,

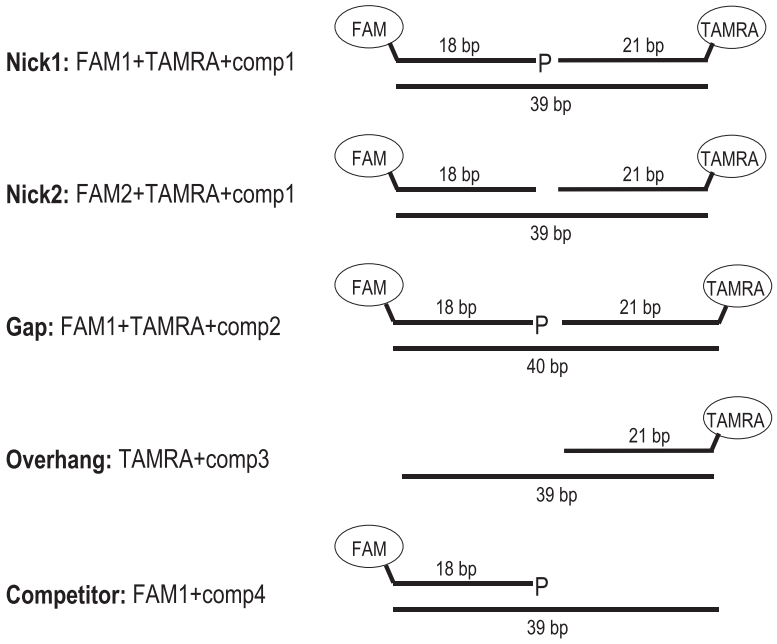


Figure 1. Schematic representation of the substrates used in this study. PAGE-purified oligonucleotides were annealed according to the scheme above to give the nicked, gapped and overhang substrates shown. The sequences (written 5′–3′) of the oligonucleotides used were FAM1: (6-FAM)TAGCATCGATCAGTCCTCp; FAM2: (6-FAM) TAGCATCGATCAGTCCTC; TAMRA: GAGGTCTAGCATCGTTAGTCA(TAMRA); COMP1: TGACTAACGATGCTAGACCTCGAGG-ACTGATCGATGCTA; COMP2: TGACTAACGATGCTAGACCTCTGAGGACTGATCGATGCTA; COMP3: TGACTAACGATGCTAGACCTCATCCGTT-CAGTACGTAGG; COMP4: GTCTAGCTAGTTGTACGAACTGAGGACTGATCGATGCTA.

Figure 2. The phosphatase activity of hPNK is faster than its kinase activity in processing a nick and a gap flanked by a 3′-phosphate and a 5′-hydroxyl. The indicated double-labelled oligonucleotide duplexes (50 nM) were incubated with wild-type hPNK at the concentrations shown for 10 min at 37°C. After quenching, the samples were resolved in a 20% denaturing polyacrylamide gel and analysed by fluorimagery.

Figure 3. Mutagenesis of the kinase domain leaves the phosphatase activity of PNK unaffected. The indicated double-labelled oligonucleotide duplexes (50 nM) were incubated with the hPNK K378A mutant at the concentrations shown for 10 min at 37°C. After quenching, the samples were resolved in a 20% denaturing polyacrylamide gel and analysed by fluorimagery.

17% glycerol and 0.1 M KCl. Extracts were aliquoted and stored at −80°C. Nicked substrate (25 nM) was incubated at 37°C with 25 μg of whole cell extracts in 50 μl reactions also containing 50 mM HEPES–KOH (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1.5 mM DTT, 2 mM ATP, 0.4 mg/ml BSA, 25 mM phosphocreatine, 2.5 μg creatine phosphokinase, 8.5% glycerol, 20 μM dNTPs, 0.2 mM NAD^+ and 1 μg of single stranded competitor DNA. Aliquots of 8 μl samples were removed at the indicated time points and quenched by addition to an equal volume of formamide loading buffer. The samples were then heated to 95°C for 3 min before loading on a 20% denaturing polyacrylamide gel. Gels were visualized by fluorimagery using a Typhoon 9410 laser scanning system (Amersham). All experiments were repeated three times and a representative gel is shown.

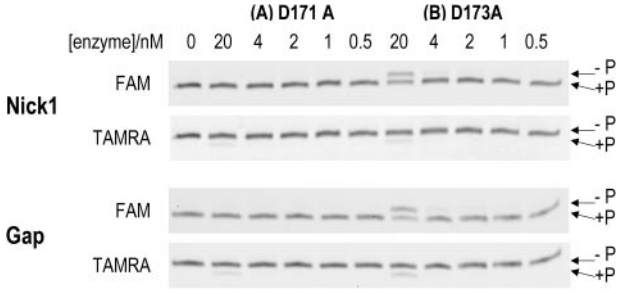
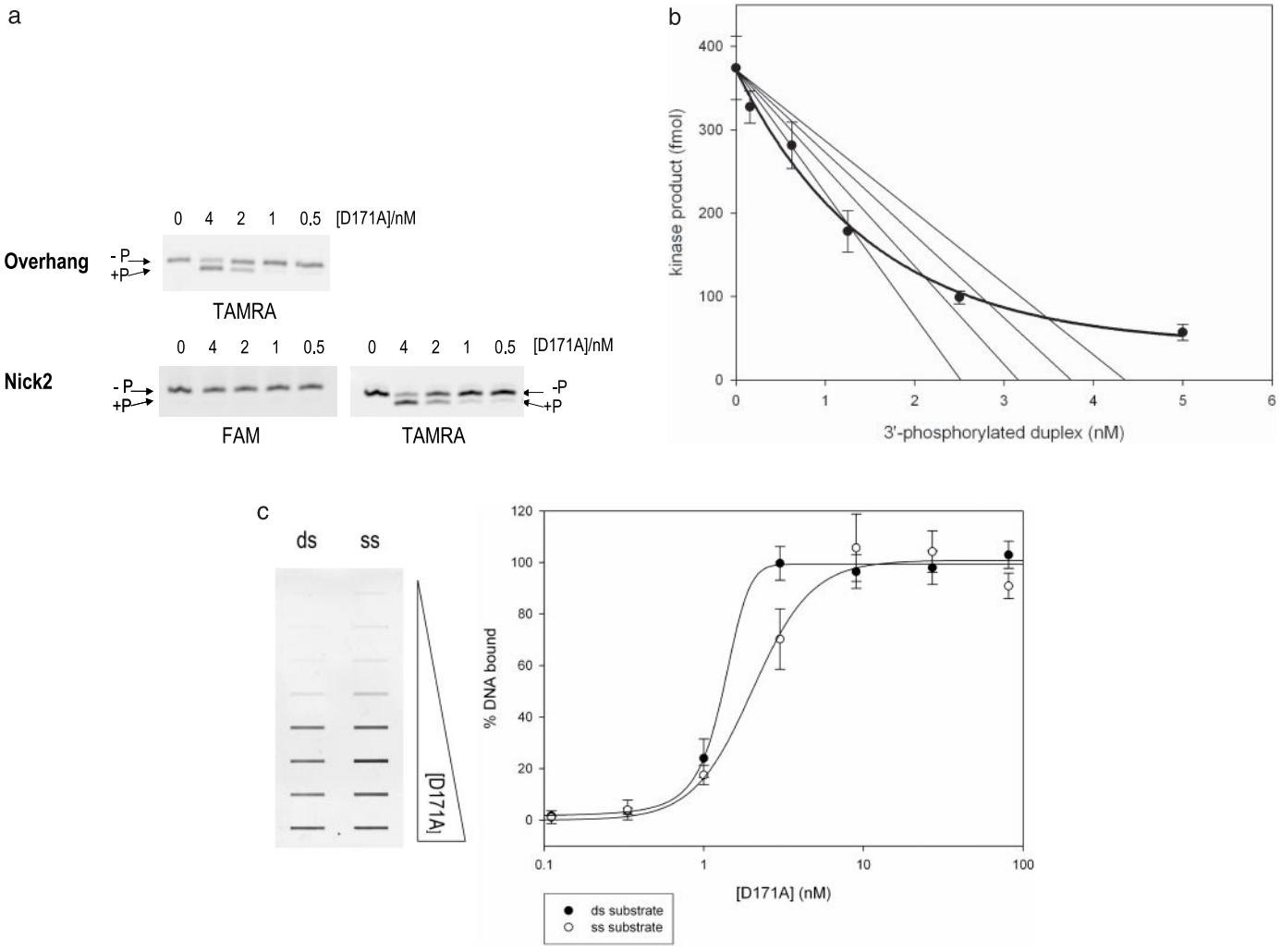


Figure 4. Mutagenesis of the phosphatase domain also abrogates the kinase function of PNK. The indicated double-labelled oligonucleotide duplexes (50 nM) were incubated with either (**A**) hPNK D171A or (**B**) hPNK D173A at the concentrations shown for 10 min at 37°C. After quenching, the samples were resolved in a 20% denaturing polyacrylamide gel and analysed by fluorimagery.

RESULTS AND DISCUSSSION

Processing of nicked and gapped substrates by hPNK

Double stranded oligonucleotide substrates were constructed containing a 3′-phosphate and 5′-hydroxyl within either a nick or a 1 nt gap and labelled at the 5′ end with FAM and at the 3′ end with TAMRA (Figure 1). Previous studies of PNK activity on substrates containing both a 5′-hydroxyl and a 3′-phosphate have used a single ^{32}P-label with the result that one of the activities can often only be inferred through the ligatability of the substrate (12,14). The use of two labels allowed us to simultaneously characterize both kinase and



phosphatase activities of hPNK for the first time. Following incubation with the enzyme, the components of the double-labelled substrate can be resolved by denaturing polyacrylamide gel electrophoresis prior to detection and quantitation by two-channel fluorimagery. Phosphatase-catalysed removal of the 3′-phosphate from the 6-FAM labelled oligonucleotide results in its decreased mobility through a denaturing polyacrylamide gel while kinase addition of a 5′-phosphate increases the mobility of the TAMRA labelled species. It has previously been demonstrated that the kinase activity of mammalian PNKs has a significant preference for 5′-hydroxyl termini associated with a nick or gap (23) and we could therefore assume minimal hPNK processing of the unlabelled complementary strand.

We initially applied this system to compare the relative activities of the phosphatase and kinase activities of hPNK in processing of SSB containing both a 5′-hydroxyl and a 3′-phosphate. Titration experiments revealed that the kinase activity of hPNK was significantly lower than its phosphatase activity on both the nicked and gapped substrates (Figure 2). While the phosphatase activity was able to process almost all the substrate within 10 min at a hPNK concentration of 1 nM, the kinase activity showed comparably little substrate turn-over at the same concentration. Since these experiments were carried out under multiple-turnover conditions it can be concluded that the kinase and phosphatase activites do not act in a concerted manner on substrates containing both a 5′-hydroxyl and a 3′-phosphate. This might be expected given the observed physical separation of the two active sites in the enzyme structure (18) but is in marked contrast to T4 PNK. The tetrameric structure of the phage enzyme, with the kinase and phosphatase domains of adjacent monomers orientated opposite each other, has been proposed to facilitate the simultaneous processing of a 5′-hydroxyl and a 3′-phosphate in the same polynucleotide (24). Since T4 PNK is a tRNA repair enzyme the differences in substrate processing between it and the mammalian enzyme are likely to reflect their differing roles *in vivo*, as recently discussed by Caldecott (25).

Disruption of the kinase active site does not affect the phosphatase activity

While the amino acid sequence of the better-characterized T4 PNK enzyme differs markedly from that of its mammalian analogues, the active sites are well-conserved in both sequence and structure (18,24,26). This conservation of active site morphology and the extensive site-directed mutagenesis studies carried out on the T4 enzyme (27,28) provided a basis for further exploration of the interdependence of the two activities of PNK by selectively mutating each in turn.

We therefore mutated the Lys 378 amino acid of hPNK to alanine to determine its importance for the kinase activity of the enzyme. Lys 378 is located in the Walker A (P loop) motif, which in the mouse enzyme has been shown to have a structural configuration identical to that found in T4 PNK (18,24). The homologous Lys 15 residue of T4 PNK was previously shown to be essential for the kinase activity of the phage enzyme (27) and is involved in ATP co-ordination (24,26). We found that Lys 378 of the human enzyme was similarly essential; with the kinase activity of the enzyme completely abolished in the K378A mutant while the phosphatase activity was unaffected (Figure 3).

Mutagenesis of the phosphatase active site disrupts both phosphatase and kinase activities

We then characterized the effects of mutating residues in the phosphatase active site on the repair activities of hPNK. The amino acids selected for mutation were Asp 171 and Asp 173, homologous respectively, to Asp 165 and Asp 167 of T4 PNK, which have previously been shown to be essential for the phosphatase activity of the T4 enzyme (27,28). While the D171A mutation showed complete abrogation of phosphatase function (Figure 4A), D173A showed residual activity at a higher enzyme concentration of 20 nM (Figure 4B). This reflects these residues' proposed differing roles in catalysis. Asp 171 is the first aspartate in the conserved Dx(D/T)x (T/V) phosphatase motif and forms the phosphoaspartate intermediate essential to the reaction mechanism. In contrast, Asp 173 has been proposed merely to aid dephosphorylation by acting as a general acid (18), protonating the alcohol leaving group and also to be involved in regeneration of the enzyme.

More unexpectedly, and in contrast to the K378A findings, we found that mutation of the phosphatase domain also significantly disrupted the kinase activity on both nicked and gapped substrates (Figure 4A and B). Despite the close association of the kinase and phosphatase domains in mammalian PNK (18) it seemed unlikely that a single amino acid change in the phosphatase active site would affect the folding of that of the kinase. These observations suggested that the kinase activity was somehow dependent on the phosphatase activity.

Figure 5. Inhibition of kinase activity of phosphatase inactive mutants by 3′-phosphorylated DNA. (**A**) hPNK mutant D171A readily phosphorylates substrates lacking a 3′-phosphate. The indicated oligonucleotide duplexes (50 nM) were incubated with hPNK D171A at the concentrations shown for 10 min at 37°C. After quenching, the samples were resolved in a 20% denaturing polyacrylamide gel and analysed by fluorimagery. (**B**) 3′-Phosphorylated oligonucleotide duplex inhibits processing of kinase substrate by D171A. Oligonucleotide duplex 'Overhang' (50 nM) was incubated with hPNK D171A (4 nM) for 8 min at 37°C in the presence of varying concentrations of 3′-phosphate-containing 'Competitor' duplex. After quenching, the samples were resolved in a 20% denaturing PAGE and analysed by fluorimagery. Data points represent the mean, and error bars the standard deviation of three independent experiments. Curve fitting was carried out by non-linear regression using SigmaPlot. The K_i is calculated as the distance between the X-intercepts of the linear plots, which represent lines drawn from V, the highest point of the curve (i.e. in the absence of inhibitor), to cross the curve at V/2, V/3, V/4, V/5 as described by Dixon (20). (**C**) Nitrocellulose filter-binding assay shows that single and double stranded 3′-phosphorylated substrates are efficiently bound by D171A. Binding reactions contained 100 pM FAM-labelled double-stranded (ds, 'Overhang', Figure 1) or single-stranded (ss, FAM1, Figure 1) substrate and concentrations of hPNK D171A ranging from 0.1–81 nM. The extent of binding was measured by nitrocellulose filter binding assay as detailed in the Materials and Methods section. Data points represent the mean, and error bars the standard deviation of three independent experiments. (**D**) Optimal inhibition is obtained with a recessed 3′-phosphate. Oligonucleotide duplex 'Overhang' (50 nM) was incubated with hPNK D171A (4 nM) for 8 min at 37°C in the presence of the indicated competitors (10 nM), where an asterisk corresponds to a 3′-phosphate. After quenching, the samples were resolved in a 20% denaturing PAGE and analysed by fluorimagery. Data points represent the mean, and error bars the standard deviation of three independent experiments.

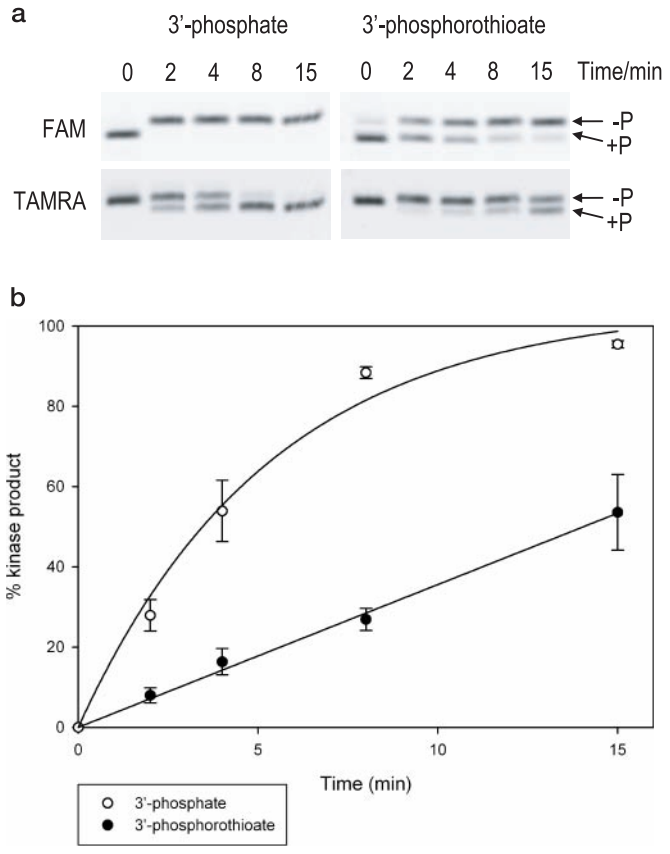


Figure 6. Wild-type PNK has inhibited kinase and phosphatase activity on a nicked substrate containing a 3′-phosphorothioate monoester. (**A**) Double-labelled nicked duplexes containing a 5′-hydroxyl and either a 3′-phosphate or a 3′-phosphorothioate monoester (50 nM) were incubated with wild-type hPNK (5 nM) for the indicated times. After quenching, the samples were resolved in a 20% denaturing polyacrylamide gel and analysed by fluorimagery. (**B**) Kinetics of hPNK kinase activity on 3′-phosphorylated or -thiophosphorylated substrates. Data points represent the mean, and error bars the standard deviation of three independent experiments.

Abrogation of kinase activity in phosphatase mutants depends on the presence of a 3′-phosphate in the substrate

To test this hypothesis we examined the ability of a phosphatase mutant to 5′-phosphorylate substrates in which a 3′-phosphate is absent. We tested the activity of the D171A mutant on a substrate containing a recessed 5′-hydroxyl with a 3′-overhang ('Overhang', Figure 1) and a substrate containing a nick flanked by both 5′- and 3′-hydroxyls ('Nick2', Figure 1). We found that the kinase activity of the D171A mutant was able to efficiently phosphorylate both of these substrates (Figure 5A). We therefore concluded that the observed disruption of the kinase activity on the previous substrates was indeed due to the presence of the 3′-phosphate. Our results also indicated that hPNK shows similar kinase processing of a 5′-hydroxyl whether located within a nick or a recess and are in good agreement with the findings of Bernstein *et al.* (18).

There are two possible models whereby the presence of a 3′-phosphate in the substrate could inhibit kinase activity: (i) the kinase domain is able to discriminate between phosphorylated and unphosphorylated substrates and only binds/processes the latter and (ii) phosphorylated substrate is bound by the phosphatase domain and on binding prevents other substrates from accessing the kinase domain. A third possibility, that the substrate is preferentially bound by the phosphatase domain and therefore cannot be accessed by the kinase domain can be discounted since these experiments were all carried out under conditions of excess substrate. In order to discriminate between these two mechanisms we examined the effects of titrating a competing substrate ('Competitor', Figure 1) containing a recessed 3′-phosphate and 5′-overhang into reactions containing the D171A mutant and the 3′-overhang substrate. If the first model were correct then the processing of the 3′-overhang substrate would be unaffected by the titration. However, if the second model were correct then addition of the competing substrate would result in inhibition of kinase activity.

The results of the titration experiments (Figure 5B) revealed the second model to be the case and that where a 5′-hydroxyl and a 3′-phosphate are both present, processing of the 3′-phosphate takes precedence. The kinase activity showed almost stoichiometric inhibition by the 3′-phosphorylated competitor. Using the graphical method developed by Dixon (20) for analysis of tight binding inhibitors (Figure 5B) and assuming mixed inhibition with a predominantly competitive component we were able to estimate the K_i for the 3′-phosphorylated competitor as being <0.6 nM. This tight binding of the phosphatase domain to its substrate is consistent with the observation that the phosphatase activity precedes that of the kinase. By comparison, the binding of the kinase domain to a sub-optimal single stranded substrate was recently found to be 1.3 μM (29).

We then compared the ability of different 3′-phosphorylated substrates to inhibit the kinase activity of the D171A mutant. We initially confirmed via nitrocellulose filter-binding experiment that hPNK mutant D171A was able to efficiently bind both single and double stranded substrates (Figure 5C). As predicted by Bernstein *et al.* (18) who showed that mammalian PNK dephosphorylates 3′-phosphorylated nicks, gaps and single stranded substrates equally well, we found that both single ($K_d \approx 1.9$ nM) and double stranded ($K_d \approx 1.2$ nM) substrates were tightly bound by the mutant enzyme. This finding correlates well with the proposed model for phosphatase substrate binding in which only the first three backbone phosphates of the substrate contribute to binding at the phosphatase surface (18).

When we tested the ability of the different phosphatase substrates to inhibit D171A kinase activity, we found that inhibition was affected by the length of the 5′-overhang, with inhibition progressively decreasing as the size of the overhang decreased (Figure 5D, compare inhibitors i–iii). Inhibitor containing a 3′-overhanging phosphate (iv) or a single stranded inhibitor (v) were both unable to substantially inhibit the kinase activity. We also confirmed that effective inhibition required the presence of a 3′-phosphate (inhibitor vi).

Taken together, these results strongly suggest that the observed kinase inhibition arises as a result of steric hindrance by the phosphatase substrate rather than through allosteric restructuring of the enzyme. Inhibitors (iv) and (v), in which the 3′-phosphate is present on single stranded DNA, will have more conformational flexibility than inhibitors (i–iii) where the 3′-phosphate is constrained at the end of a

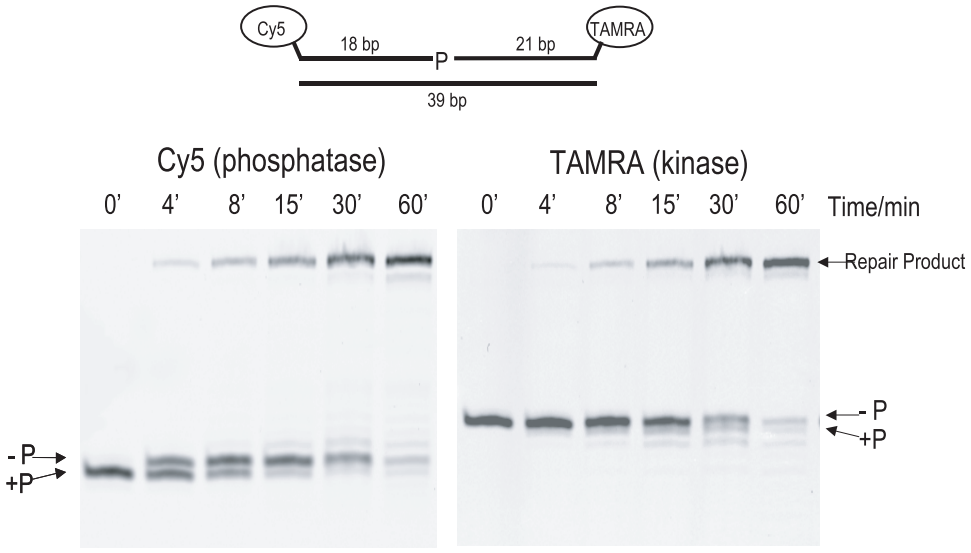


Figure 7. The kinase activity of PNK is rate-limiting in the repair of a nick flanked by a 3′-phosphate and a 5′-hydroxyl. Double-labelled oligonucleotide duplex (25 nM) was incubated with 25 μg of HeLa S3 whole cell extract at 37°C for the indicated times. After quenching, the samples were resolved in a 20% denaturing polyacrylamide gel and analysed by fluorimagery.

double helix. This flexibility presumably allows substrate to access the kinase active site.

The phosphatase activity of the wild-type enzyme also precedes the kinase

It was important to determine whether the results obtained for the phosphatase inactive mutant could be extrapolated to the wild-type enzyme. If so, it might be expected that a nick containing a 3′-phosphate would be phosphorylated by the kinase activity less efficiently than a nick without a phosphate. In practice however, we found that the phosphatase activity of hPNK was so much faster than the kinase activity that the 3′-phosphorylated nick was effectively transformed into an unphosphorylated nick and so no difference in kinase activity was observable (data not shown).

We therefore employed a modified nicked substrate, substituting the 3′-phosphate with a 3′-phosphorothioate monoester in which one of the non-bridging phosphate oxygens has been replaced with sulfur. While this is a fairly conservative change structurally, phosphorothioates show important differences in reactivity compared to phosphates: the negative charge localizes to the sulfur and the phosphorus is less electrophilic. Accordingly, it has been shown that thiophosphorylated proteins are relatively resistant to dephosphorylation by protein phosphatases when compared to normal phosphoproteins (30).

We treated the 3′-phosphorothioate nick with wild-type hPNK and found that the phosphatase activity indeed hydrolysed the phosphorothioate more slowly than the corresponding phosphate (Figure 6A, FAM panels), presumably due to less ready formation of the phosphoaspartate intermediate with Asp 171. This decrease in phosphatase activity was accompanied by a decrease in kinase activity (Figure 6A, TAMRA panels and 6B) and therefore recapitulated the mutagenesis results. It can thus be concluded that 5′-phosphorylation of strand breaks containing a 3′-phosphate and 5′-hydroxyl depends on prior processing by the phosphatase activity of hPNK.

The kinase activity of hPNK is rate-limiting in cell extract-mediated repair

Our results suggested that the kinase activity of hPNK might be rate-limiting in the *in vivo* repair of nicks containing 3′-phosphate and 5′-hydroxyl. However, it is known that PNK-dependent repair of SSB is stimulated by the presence of other repair factors, such as XRCC1 (14) and Ligase I (12). We therefore examined the repair of a nicked substrate by HeLa whole cell extracts (Figure 7), in this case substituting a Cy5 label for the 6-FAM label previously used, since preliminary experiments revealed 6-FAM to be labile in cell extracts. We found that while the phosphatase product accumulated during the course of the reaction the kinase product did not and was instead immediately processed to yield the double-labelled 39 bp product of strand ligation (Figure 7). We therefore concluded that the kinase activity of PNK is indeed rate-limiting in the repair of this type of strand break.

Strand breaks with 3′-phosphate and 5′-hydroxyl termini arise through the Tdp1 processing of abortive topoisomerase I complexes, such as are generated by the anti-neoplastic drug camptothecin. A role for PNK in processing camptothecin-induced DNA damage has long been proposed (2) and was recently demonstrated by RNAi knockdown of hPNK (16). It was also recently shown that hPNK is present *in vivo* as part of a multi-enzyme complex with Tdp1(15). Our results suggest that hPNK mediated phosphorylation of the 5′-hydroxyl terminus would be a key modulating factor in determining the efficiency of repair of these lesions.

The reason for giving precedence to the phosphatase domain over the kinase domain may lie in the relative importance of the two activities in protecting against DNA damage. Ionising radiation, for example, generates substantially more 3′-phosphate termini than 5′-hydroxyl termini. Similarly, the

PNK-dependent sub-pathway of BER initiated by bifunctional glycosylases requires only the phosphatase activity of PNK (3). Furthermore, while a 3′-phosphate completely blocks repair synthesis by being non-processible by both DNA polymerases and ligases, a 5′-hydroxyl only blocks ligation and could theoretically be bypassed by extending DNA synthesis. Although it has been reported that the optimal substrate for the repair polymerase Pol β is a 1 nt gap containing a 5′-phosphate (31), it is possible that it or another polymerase could displace the 5′-hydroxyl in a flap, which could then be excised by FEN-1 in a mechanism analogous to that employed in the long-patch sub-pathway of base excision repair (32).

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