XRCC1 Stimulates Polynucleotide Kinase by Enhancing Its Damage Discrimination and Displacement from DNA Repair Intermediates

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Human polynucleotide kinase (hPNK) is required for processing and rejoining DNA strand break termini. The 5'-DNA kinase and 3'-phosphatase activities of hPNK can be stimulated by the “scaffold” protein XRCC1, but the mechanism remains to be fully elucidated. Using a variety of fluorescence techniques, we examined the interaction of hPNK with XRCC1 and substrates that model DNA single-strand breaks. hPNK binding to substrates with 5'-OH termini was only ~5-fold tighter than that to identical DNA molecules with 5'-phosphate termini, suggesting that hPNK remains bound to the product of its enzymatic activity. The presence of XRCC1 did not influence the binding of hPNK to substrates with 5'-OH termini, but sharply reduced the interaction of hPNK with DNA bearing a 5'-phosphate terminus. These data, together with kinetic data obtained at limiting enzyme concentration, indicate a dual function for the interaction of XRCC1 with hPNK. First, XRCC1 enhances the capacity of hPNK to discriminate between strand breaks with 5'-OH termini and those with 5'-phosphate termini; and second, XRCC1 stimulates hPNK activity by displacing hPNK from the phosphorylated DNA product.

Scission of the DNA sugar-phosphate backbone is a common form of damage that can be induced not only by a broad range of genotoxic agents, but also as an intermediate product in several DNA repair pathways. The term “strand break” covers an array of diverse chemical structures. Aside from single- and double-strand breaks, there are many chemically distinct end groups found at strand break termini. Repair of these strand interruptions is usually mediated by DNA polymerases and ligases. All DNA polymerases and ligases characterized to date are highly selective for the type of DNA ends that can be utilized. Both of these classes of enzymes require 3'-hydroxyl DNA termini, and the DNA ligases also require 5'-phosphate termini. However, the termini generated by several endonucleases, as well as those induced by ionizing radiation, frequently bear 5'-hydroxyl and/or 3'-phosphate groups (1–5) and must therefore be processed before they can be acted upon by DNA ligases or polymerases.

Mammalian polynucleotide kinase (PNK)3/phosphatase is a bifunctional enzyme that can phosphorylate 5'-OH termini and dephosphorylate 3'-phosphate termini of DNA (6, 7). It is a DNA repair enzyme involved in the processing of strand break termini to a form suitable for other proteins to complete the replacement of missing nucleotides and strand rejoining (8–11). PNK is implicated in the repair of both single-strand breaks (SSBs) and double-strand breaks. PNK stimulates SSB repair in both in vitro reconstitution experiments (5, 10, 12, 13) and in vivo studies (14). Further evidence has indicated that, as a result of its required involvement in the repair of specific types of SSBs, PNK participates in the base excision repair pathway following the formation of strand breaks induced by DNA glycosylases such as NEIL1 and NEIL2 (5) and in the repair of topoisomerase-1 “dead-end” complexes (15, 16). PNK is also an integral component of the non-homologous end-joining pathway, which is the major route for the repair of double-strand breaks in mammalian cells (17–19).

An important feature of mammalian PNK (which distinguishes it from T4 phage PNK) is that it acts efficiently at sterically hindered internal strand breaks, a prerequisite for an enzyme involved in SSB repair. Indeed, previous studies demonstrated that PNK preferentially phosphorylates internal or recessed 5'-OH termini in comparison with 5'-OH termini on single-stranded DNA or at overhanging or blunt double-stranded ends (10, 11). The crystal structure of murine PNK suggests that this may be due to increased contact between enzyme and substrate afforded by conserved positively charged residues on the surface of the protein (20). One purpose of this study was to determine the binding affinity of human (h) PNK for model ligands representing SSBs with either a nick or a single-nucleotide gap to correlate its binding affinity to its kinase activity. We made use of a fluorescence approach that we

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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3 The abbreviations used are: PNK, polynucleotide kinase; SSBs, single-strand breaks; h, human; FRET, fluorescence resonance energy transfer; AC, acrylodan.
previously employed to examine the interaction between hPNK and a single-stranded DNA substrate, which revealed that hPNK forms a ternary complex with DNA and ATP, thereby providing evidence supporting a sequential reaction mechanism for the phosphorylation of DNA (21).

The XRCC1 (x-ray repair cross-complementing group 1) protein is also required for DNA SSB repair and genetic stability, although its biochemical role has yet to be fully established. Current evidence strongly suggests that XRCC1 functions as a chaperone or scaffolding protein capable of interacting with several proteins participating in SSB repair. It forms repair complexes with poly(ADP-ribose) polymerase-β (24, 25), DNA polymerase-β (24, 25), DNA ligase III (26), human apurinic/apyrimidinic endonuclease (27), and proliferating cell nuclear antigen at DNA replication foci to facilitate SSB repair during S phase (28). XRCC1 also binds PNK through the PNK forkhead-associated domain and stimulates the DNA kinase and DNA phosphatase activities at damaged DNA termini (13, 14). However, the mechanism underlying this stimulation remains to be characterized. Here we report our studies on the interaction between hPNK and XRCC1 in the absence and presence of several substrates that model DNA strand breaks. Our results indicate that a major function of XRCC1 is to stimulate hPNK activity by displacing hPNK from the phosphorylated DNA product.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Recombinant hPNK and His-tagged XRCC1 were purified as described previously (21, 29). The oligonucleotide substrates (5′-phosphorylated and non-phosphorylated) were synthesized by University Core DNA Services (University of Calgary, Calgary, Alberta, Canada), and their sequences are provided in Table 1.

The hPNK single mutants C409A and C437A were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Each reaction contained 50 ng of template pBluescript SK+/hPNK and 125 ng of sense and antisense primers. Eighteen PCR cycles were performed using an extension temperature of 65 °C. No further modifications were made to the protocol. The plasmids were sequenced on an ABI 310 genetic analyzer. The hPNK mutants were subcloned from the cloning vector into pET16b (Novagen) for expression.

**Labeling of hPNK with 6-Acryloyl-2-dimethylamino-naphthalene**—hPNK was dissolved in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, and 1 mM dithiothreitol and dialyzed overnight at 4 °C against the same buffer. Subsequently, dialysis was performed overnight at 4 °C against 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM MgCl₂ without dithiothreitol. Acrylodan (AC; Molecular Probes, Eugene, OR) was dissolved in N,N-dimethylformamide, and the mixture was added slowly until an ~3-fold molar excess of reagent to protein was achieved. The reaction was allowed to proceed for 2 h at 4 °C in the dark on a mechanical rocker. The reaction mixture was centrifuged at 15,000 × g for 10 min to remove the precipitated AC, and the supernatant was subjected to exhaustive dialysis with several changes against 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM MgCl₂, to remove traces of excess AC. The extent of labeling of the protein with AC was quantified by independent determinations of the amounts of AC and protein in the reaction mixture. The AC concentration was determined spectrophotometrically using an absorption coefficient of 1.64 × 10⁴ M⁻¹ cm⁻¹ at 380 nm (30, 31). The concentration of AC-labeled protein was determined using the Bradford assay (32).

**Steady-state Fluorescence Studies**—Steady-state fluorescence spectra were measured at 25 °C on a PerkinElmer Life Sciences LS-55 spectrofluorometer with 5 nm spectral resolution for excitation and emission using 0.1–0.2 μM solutions of purified hPNK as described in our previous studies (29, 33). Protein fluorescence was excited at 295 nm, and fluorescence emission spectra were recorded in the 300–400-nm range; changes in fluorescence were usually monitored at the emission maximum (340 nm). In studying the effects of substrates, we made additions to reaction mixtures from stock solutions, keeping the dilution below 3%, and fluorescence intensities were corrected for dilution factors. The total absorption of protein samples was kept below 0.05 at 295 nm. In the case of the AC-labeled hPNK protein (referred to as hPNK-AC), excitation was at 380 nm, and the changes in AC fluorescence at the emission maximum (495 nm) were monitored. For fluorescence resonance energy transfer (FRET) analysis, proteins were excited at 295 nm, and emission was measured at 495 nm.

**Fluorescence Lifetime Measurements**—Fluorescence intensity decay measurements were performed using an EasyLife LS fluorescence lifetime system (Photon Technology International, Inc., Lawrenceville, NJ). hPNK-AC was excited at 380 nm using a pulsed NanoLED system capable of measuring lifetimes from 100 ps. Fluorescence emissions were monitored at 495 nm. The instrument response function was determined by scattering the excitation light with a dilute solution of nondairy coffee creamer. Decay curves were averaged from five acquisitions. All experiments were performed at 25 °C. The fluorescence decay curves were analyzed by a reconvolution procedure using a nonlinear regression program (Photon Technology International, Inc.). Good curve fits for hPNK-AC and complexes (hPNK-AC plus substrates) were obtained for a single lifetime component. The χ² values of good curve fits (obtained using the Felix32 software program provided by the instrument manufacturer) ranged between 0.85 and 1.1.

**Kinase Activity Assay**—Two 50-μl reaction mixtures containing kinase buffer (80 mM succinic acid (pH 5.5), 10 mM MgCl₂, and 1 mM dithiothreitol), 0.2 nmol of DNA substrate, 0.4 nmol of unlabeled ATP, 3.3 pmol of [γ-32P]ATP, and 500 ng of hPNK were incubated at 37 °C. From one of the reaction mixtures were taken 3-μl samples after incubation intervals of 0, 1, 2, 5, 10, 20, and 30 min. To the other reaction mixture was added 4.0 μg of XRCC1 after a 20-min incubation, and 3-μl samples were taken after 21-, 22-, 25-, 30-, 40-, and 50-min incubations. The samples were mixed with sequencing gel loading dye (Fisher) and run on a 7 M urea-containing 12% polyacrylamide sequencing gel at 200 V. Gels were scanned on a Typhoon 9400 variable mode imager (GE Healthcare, Bucks, UK), and the resulting bands were quantified using ImageQuant 5.2 (GE Healthcare).
RESULTS

Overview of the Fluorescence-based Analytical Approach—A series of steady-state and lifetime fluorescence measurements were carried out to study the interaction between hPNK, its DNA substrates, and XRCC1. Binding of hPNK to substrates was examined by monitoring the influence of the substrates on the intrinsic Trp fluorescence of hPNK. For studies involving hPNK and XRCC1, hPNK was labeled with AC, a sulfhydryl-specific covalent label, and the influence of XRCC1 binding to hPNK-AC was monitored by quenching of AC fluorescence at 495 nm following direct excitation of AC at 380 nm and by FRET experiments. FRET is a powerful technique used to study conformational changes in proteins following protein/protein and protein/ligand interactions (33, 34). For the FRET experiments, hPNK-AC was excited at 295 nm, and the effect of interactions with DNA, XRCC1, and the DNA/XRCC1 complexes on the Trp-sensitized AC fluorescence at 495 nm was determined (i.e. monitoring the effect of ligand binding on the efficiency of energy transfer). Fluorescence lifetime experiments were carried out to further characterize the various fluorescent complexes of hPNK-AC formed upon binding DNA and XRCC1.

Binding Affinities of hPNK for Substrates That Model DNA Strand Breaks—Active recombinant hPNK was produced in E. coli and purified as described previously (21). Steady-state fluorescence spectroscopy was employed to study the effects of the binding of model ligands representing SSBs with either a nick or a single-nucleotide gap, the intact duplex with no break, and a single-stranded oligonucleotide (24-mer) on hPNK fluorescence. Duplex structures modeling nicked and gapped substrates were synthesized with 5'-phosphate groups at their double-strand termini, which we refer to here as external phosphates (see Table 1 for sequences). The observed intrinsic fluorescence was due to Trp residues excited at 295 nm. hPNK/ligand interactions resulted in partial quenching of hPNK fluorescence with no change in emission maximum, which allowed determination of binding affinities ($K_d$) by following fluorescence quenching as a function of ligand concentrations (21). A representative plot of relative fluorescence intensities versus the concentrations of nicked DNA is shown in Fig. 1A (inset). Nonlinear regression analysis (GraphPad Prism software) of the binding data gave a best fit for a two-site model, and the $K_d$ values obtained were $0.25 \pm 0.03$ and $1.30 \pm 0.20 \mu M$ for the internal nick (5'-OH) and for the external duplex ends (5'-

### TABLE 1

| Model substrate          | Oligonucleotide sequences |
|--------------------------|---------------------------|
| Nicked DNA               | 5'-pATTACGAATGCCCACACCGCCGGCGCCCACCACACTAGCTGGCC-3' |
| Nicked DNA (5'-P)        | 5'-pATTACGAATGCCCACACCGCCGGCGCCCACCACACTAGCTGGCC-3' |
| 1 nt-gapped DNA          | 5'-pATTACGAATGCCCACACCGCCGGCGCCCACCACACTAGCTGGCC-3' |
| 24-mer                   | 5'-pGGCGCCCACACCGCCGGCGCCCACCACACTAGCTGGCC-3' |
| 24-mer (5'-P)            | 5'-ATTACGAATGCCCACACCGCCGGCGCCCACCACACTAGCTGGCC-3' |
| Duplex                   | 5'-pGGCGCCCACACCGCCGGCGCCCACCACACTAGCTGGCC-3' |

*a nt, nucleotide.
phosphate), respectively (Table 2). Similar results were obtained with the gapped substrate. We also measured the binding of the nicked DNA with an internal 5′-phosphate to hPNK (Fig. 1B). The 5′-phosphate nicked DNA, which can be considered a product of the enzymatic reaction of PNK, showed lower affinity for hPNK, as would be anticipated, and the binding data revealed unimodal binding with a $K_d$ of 1.4 ± 0.2 μM, i.e. all three phosphates (one internal and two external) displayed similar affinities for hPNK. The $K_d$ values obtained with all of the substrates are presented in Table 2. The affinities with which hPNK bound these ligands were 1 nucleotide-gapped DNA > nicked DNA > intact duplex > 5′-phosphate nicked DNA. The 5′-OH single-stranded oligonucleotide (24-mer) exhibited higher affinity than the 5′-phosphate single-stranded oligonucleotide (24-mer). The observed values indicate that there is a modest but significant difference in binding affinities for substrates with 5′-OH versus 5′-phosphate DNA and also for double-stranded versus single-stranded oligonucleotides.

**Acrylodan Labeling of hPNK**—Because both hPNK and XRCC1 have Trp and Tyr residues, one cannot easily study the interaction between these two proteins using the intrinsic fluorescence of Tyr and Trp residues. For this reason, we used the covalent sulfhydryl-specific environment-sensitive fluorescent probe AC to label hPNK (30, 31). hPNK-AC was functionally active when tested for its kinase activity and retained probe AC to label hPNK (30, 31). hPNK-AC was functionally active when tested for its kinase activity and retained probe AC to label hPNK (30, 31). hPNK-AC was functionally active.

**Interaction of XRCC1 with Polynucleotide Kinase**—The interaction between hPNK-AC and XRCC1 was studied by monitoring the XRCC1-induced perturbation of the labeled AC environment following excitation of hPNK-AC at 380 nm. The hPNK-AC/XRCC1 interaction was accompanied by partial quenching of AC fluorescence with no significant change in the emission maximum, which allowed determination of the binding affinity ($K_d$) and stoichiometry by following fluorescence quenching (a measure of XRCC1 binding) as a function of XRCC1 concentration. A plot of relative fluorescence intensities versus concentration of XRCC1 is shown in Fig. 2A (inset). The maximum quenching of fluorescence intensity observed (∼45%) at a saturating concentration of XRCC1 was normalized to 1, and the observed quenching at different concentrations of XRCC1 was plotted as the fraction bound versus free XRCC1 concentration (Fig. 2A). Nonlinear regression analysis (GraphPad Prism software) of the binding data revealed unimodal binding with a $K_d$ of 30 ± 2 nm. In Fig. 2B, $ΔF/ΔF_{max}$ is plotted against [hPNK-AC]/[XRCC1] to determine the binding stoichiometry, and as can be seen, the $ΔF$ value leveled off when the molar ratio was 1:1, taking the monomer molecular masses of 57,100 and 69,525 Da for hPNK-AC and XRCC1, respectively. The ability of hPNK-AC to bind XRCC1 in the presence of nicked DNA was studied by initially adding nicked DNA to hPNK-AC at a 1:1 molar ratio, taking the observed fluorescence intensity at 495 nm (following excitation at 380 nm) as the starting control value, and then titrating this binary complex with XRCC1 as before. The maximum quenching obtained was ∼50%, similar to the value obtained in the absence of nicked DNA. In addition, the $K_d$ was 27 ± 2 nm, and the binding stoichiometry was 1:1, suggesting that the presence of nicked DNA did not interfere with hPNK-AC.XRCC1 interaction (data not shown).

**Evidence for Different hPNK Binding Sites for DNA and XRCC1**—The interaction between hPNK-AC and its substrates that model DNA strand breaks was monitored by following changes in AC fluorescence at 495 nm after excitation at 295 nm, a measure of substrate-induced Trp-sensitized AC fluorescence. There was good overlap between the emission spectrum of hPNK and the absorption spectrum of hPNK-AC, thus meeting the requirement for efficient FRET from the donor Trp to the acceptor AC (Fig. 3). When hPNK was excited at 295 nm, the emission maximum was centered at 340 nm. Addition of AC quenched Trp fluorescence at 340 nm, and this was accompanied by the appearance of AC fluorescence at 495 nm.

### Table 2

| Substrate                                | $K_d$ (μM) | $K_f$ (μM) |
|------------------------------------------|------------|------------|
| 1 nt-gapped DNA                          | 0.20 ± 0.03| 1.20 ± 0.2 |
| Nicked DNA                               | 0.25 ± 0.03| 1.30 ± 0.2 |
| Nicked DNA (5′-P)                        | 1.40 ± 0.20|            |
| Duplex (45-mer)                          | 0.50 ± 0.10|            |
| Single-stranded oligonucleotide (24-mer) | 1.05 ± 0.10|            |
| Single-stranded oligonucleotide (24-mer, 5′-P) | 3.80 ± 0.30|            |

* $5′$-P indicates the presence of a phosphate at the DNA terminus.
* Duplex contains one 5′-OH and one 5′-phosphate terminus.
Because free AC does not fluoresce under these experimental conditions, the observed peak at 495 nm represented Trp-excited AC fluorescence and demonstrated energy transfer to AC.

The emission spectrum of hPNK-AC when excited at 295 nm is shown in Fig. 4. Addition of nicked DNA quenched AC fluorescence at around 495 nm, and the subsequent addition of XRCC1 produced further quenching. The binding affinity of hPNK-AC for nicked DNA was determined from fluorescence titration as a function of nicked DNA concentration by following changes in AC fluorescence at 495 nm upon excitation at 380 nm. The \( K_d \) obtained was 0.27 ± 0.04 \( \mu M \), which is in good agreement with the \( K_d \) obtained by monitoring the quenching of intrinsic fluorescence at 340 nm following excitation of Trp at 295 nm in unlabeled hPNK (Table 2). Hence, AC labeling did not affect binding of nicked DNA to hPNK. We also studied binding of nicked DNA to hPNK-AC in the presence of XRCC1. For this experiment (Fig. 4A), we initially added XRCC1 to hPNK-AC at a 1:1 molar ratio, taking the observed fluorescence intensity at 495 nm as the starting control value, and we then monitored the effect of addition of nicked DNA on fluorescence intensity at 495 nm as a function of concentration. The \( K_d \) obtained was 0.30 \( \mu M \), indicating that nicked DNA was capable of binding to hPNK-AC in the presence of XRCC1 (data not shown). In another experiment (Fig. 4B), nicked DNA was first added to hPNK-AC, followed by addition of XRCC1. As can be seen, the order of addition of XRCC1 and nicked DNA had no significant effect on the final emission spectrum, suggesting that XRCC1 and nicked DNA bound to different sites on hPNK-AC, but sufficiently close to the primary labeled Cys residue to perturb its environment. The observed quenching of AC fluorescence by nicked DNA was significantly higher compared with that by XRCC1, and this could be a reflection of their respective distances from the AC-labeled Cys residue.

**hPNK/XRCC1/DNA Interactions**—The observed quenching of the FRET signal upon binding of substrates and/or XRCC1 was significantly higher than quenching following direct excitation of hPNK-AC at 380 nm, and for that reason, FRET analysis was utilized in subsequent studies involving binary and ternary complexes. Binding of XRCC1 perturbs the FRET signal of hPNK-AC, and this can be utilized to obtain a binding constant for the interaction of the two proteins. The binding affinity (\( K_d \)) obtained by fluorescence titration was 31 ± 2 \( \mu M \), and the stoichiometry was 1:1, the same value that was obtained from direct excitation of AC at 380 nm (Fig. 2).

The extent of quenching induced by various combinations of protein and substrates is reported in Table 3. For this study, hPNK-AC and XRCC1, when present together, were in equimolar ratio at concentrations of 0.2 \( \mu M \). A 2-fold excess of each DNA substrate was added to study the effect on hPNK-AC in the absence and presence of XRCC1. Binding of nicked DNA to hPNK-AC produced ~18% quenching of fluorescence intensity, and nicked DNA also bound to hPNK-AC in the presence of XRCC1 (i.e. to the hPNK-AC-XRCC1 binary complex), inducing ~16% quenching after normalizing for the effect of XRCC1. Thus, the presence of XRCC1 had no significant effect on hPNK-AC binding to nicked DNA. hPNK-AC also bound to XRCC1 in the absence or presence of nicked DNA (11 and 12% quenching, respectively). Collectively, these data clearly demonstrate that hPNK-AC could bind to nicked DNA and XRCC1 in the presence of each other and form a ternary complex.
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The labeled protein was excited at 295 nm, and the effects of substrate binding on the AC fluorescence at 495 nm were monitored. The decrease in total fluorescence intensity at 495 nm is reported as percent quenching. Values represent means ± S.E. (n = 3).

| Complex                              | Quenching |
|--------------------------------------|-----------|
| hPNK · nicked DNA                    | 18 ± 2    |
| hPNK · XRCC1 + nicked DNA            | 16 ± 2    |
| hPNK · XRCC1                         | 11 ± 1    |
| hPNK · nicked DNA + XRCC1            | 12 ± 1    |
| hPNK · nicked DNA (5′-P)              | 12 ± 1    |
| hPNK · XRCC1 + nicked DNA (5′-P)      | 2 ± 1     |
| hPNK + XRCC1 · nicked DNA            | 12 ± 1    |
| hPNK + XRCC1 · nicked DNA (5′-P)      | 2 ± 1     |
| hPNK · nicked DNA (5′-P) + XRCC1      | 8 ± 2     |
| hPNK · 24-mer (5′-OH)                 | 16 ± 2    |
| hPNK · XRCC1 + 24-mer (5′-OH)         | 13 ± 2    |
| hPNK · 24-mer (5′-OH) + XRCC1         | 12 ± 1    |
| hPNK · 24-mer (5′-P)                  | 10 ± 2    |
| hPNK · XRCC1 + 24-mer (5′-P)          | 2 ± 1     |
| hPNK + XRCC1 · 24-mer (5′-P)          | 12 ± 1    |
| hPNP + XRCC1 · 24-mer (5′-P)          | 2 ± 1     |
| hPNK · 24-mer (5′-P) + XRCC1          | 7 ± 2     |

* A 2-fold excess of substrate was added to hPNK-AC. Labeled hPNK and XRCC1 were in equimolar concentrations.

FIGURE 4. Fluorescence emission spectra of hPNK-AC. A, shown are the fluorescence emission spectra of hPNK-AC alone (trace 1), hPNK-AC + nicked DNA (trace 2), and hPNK-AC + nicked DNA + XRCC1 (trace 3). B, XRCC1 was first added to hPNK-AC followed by nicked DNA to obtain the fluorescence emission spectra of hPNK-AC alone (trace 1), hPNK-AC + XRCC1 (trace 2), and hPNK-AC + XRCC1 + nicked DNA (trace 3). The concentration of hPNK-AC and XRCC1 used was 0.2 μM, and that of nicked DNA was 1 μM. The excitation wavelength was 295 nm.

FIGURE 5. Time-resolved fluorescence decay curves of hPNK-AC. ○, hPNK-AC; □, hPNK-AC + nicked DNA (5′-phosphate); ▽, hPNK-AC + nicked DNA (5′-phosphate) + XRCC1. The smooth lines are the best fit curves. Excitation was at 380 nm, and emission was monitored at 495 nm. Rel. fluor., relative fluorescence.

By comparison, although addition of nicked DNA (5′-phosphate) to hPNK-AC resulted in 12% quenching, no significant additional quenching was observed when the nicked DNA (5′-phosphate) was added to the hPNK-AC-XRCC1 binary complex. In other words, when XRCC1 was bound to hPNK, the nicked DNA (5′-phosphate) was not able to bind to hPNK and remained free or was bound to XRCC1 in such a way that it did not perturb the AC fluorescence of hPNK-AC. The XRCC1-nicked DNA binary complex formed upon mixing at a 1:1 molar ratio was capable of binding to hPNK-AC and produced 12% additional quenching of AC fluorescence, thus providing evidence for the formation of a ternary complex. However, the complex formed by mixing XRCC1 with nicked DNA (5′-phosphate) at a 1:1 molar ratio had no significant effect on AC fluorescence of hPNK-AC, again providing no evidence for a ternary complex. On the other hand, addition of XRCC1 to the hPNK-AC-nicked DNA (5′-phosphate) binary complex resulted in ~8% net quenching, indicating the formation of a ternary complex and suggesting that the order of addition is important. Because nicked DNA but not the product (5′-phosphate nicked DNA) was capable of binding to hPNK-AC pre-bound to XRCC1, one may infer that (i) 5′-OH and 5′-phosphate nicked DNAs probably bind at different sites on hPNK-AC and that (ii) the observed difference in their effects could be due to differences in steric hindrance arising from the presence of XRCC1. Similar but consistent results were obtained when we used the single-stranded 24-mers with 5′-OH and 5′-phosphate.

Fluorescence Lifetime Measurements—Time-resolved fluorescence decay curves for hPNK-AC, the hPNK-AC-nicked DNA (5′-phosphate) complex, and the hPNK-AC-nicked DNA (5′-phosphate) complex + XRCC1 are shown in Fig. 5. Assuming the decay curves to be monoexponential yielded a good fit in every case, and the χ² values obtained ranged from 0.88 to 1.1. The labeled protein hPNK-AC had a fluorescence lifetime (τ) of ~3.70 ns (Table 4), which is consistent with the values reported for AC bound to other proteins (35, 36). The lifetime for the hPNK-AC-nicked DNA (5′-phosphate) binary complex increased to 4.50 ns, which is indicative of conformational changes in hPNK-AC as a result of complex formation affecting the environment of AC. However, upon addition of XRCC1 to this binary complex, the lifetime value obtained was 3.80 ns, suggesting the release of nicked DNA (5′-phosphate) from hPNK-AC because addition of XRCC1 to hPNK-AC by itself had no significant effect on the hPNK-AC lifetime. On the other hand, the lifetime of the hPNK-AC-nicked DNA (5′-OH) binary complex (4.40 ns) was altered by addition of XRCC1 (τ = 4.75 ns), suggesting the presence of a ternary complex involving hPNK-AC, nicked DNA (5′-OH), and XRCC1. Similar results were also obtained with the 24-mer with 5′-OH and 5′-phosphate termini. In experiments in which XRCC1 was added to hPNK-AC first, only nicked DNA (5′-OH) and the 24-mer (5′-OH) were able to bind to this binary complex and alter its life-
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TABLE 4

Effect of substrates and XRCC1 binding on the fluorescence lifetime of hPNK-AC

A 2-fold excess of substrate was added to hPNK-AC. Labeled hPNK and XRCC1 were in equimolar concentration.

| State                        | Fluorescence lifetimes | ns | r (±0.10) | χ² |
|------------------------------|------------------------|----|-----------|----|
| hPNK                         |                        |    | 3.70      | 0.97|
| hPNK + nicked DNA (5'-P)     |                        |    | 4.50      | 1.07|
| hPNK + nicked DNA (5'-P) + XRCC1 |                    |    | 3.80      | 1.05|
| hPNK + nicked DNA            |                        |    | 4.40      | 0.87|
| hPNK + nicked DNA + XRCC1    |                        |    | 4.75      | 0.94|
| hPNK + XRCC1                 |                        |    | 3.80      | 0.90|
| hPNK + XRCC1 + nicked DNA (5'-P) |                    |    | 4.55      | 0.96|
| hPNK + XRCC1 + nicked DNA    |                        |    | 3.85      | 0.90|
| hPNK + XRCC1 - nicked DNA    |                        |    | 4.35      | 1.07|

FIGURE 6. Kinetics of product accumulation. A, with nicked DNA; B, with the single-stranded oligonucleotide (24-mer, 5'-OH). The concentration of nicked DNA and the 24-mer (5'-OH) was 4 μM, and the hPNK concentration was 0.17 μM. At 20 min of incubation, 1.1 μM XRCC1 was added. The assays were carried out four times, and a representative plot is shown.

time and not the phosphorylated products, nicked DNA (5'-phosphate) and the 24-mer (5'-phosphate).

Influence of XRCC1 on the Turnover of hPNK—The results of our fluorescence studies suggested that binding of XRCC1 resulted in the release of the product from hPNK. For this reason, we wanted to look at the effect of XRCC1 on the turnover of hPNK. The kinase activity of hPNK was assayed using a limited concentration of enzyme with nicked DNA and the single-stranded oligonucleotide (24-mer) as model DNA substrates (Fig. 6, A and B, respectively). The rate of product accumulation decreased over the course of the assay and reached a plateau in ~10 min. Addition of XRCC1 at 20 min (i.e. in this plateau region) resulted in reactivation of kinase activity, and the percent 32P incorporated nearly doubled in both instances. The observed increase in kinase activity was due to hPNK because XRCC1 itself has no kinase activity (13). Thus, addition of XRCC1 altered the turnover rate of the enzyme-product adduct. Because XRCC1 binds the substrates and products of nicked DNA and the single-stranded oligonucleotide (24-mer) with high affinity (29), it was able to release the product (bound phosphorylated substrate) from hPNK, thus freeing the enzyme to continue its enzymatic activity.

DISCUSSION

Many of the individual components involved in DNA repair pathways have been identified. There is now a major emphasis on elucidating the step-by-step choreography of each process, including the nature of the binding of repair proteins to their DNA substrates and to each other. The purpose of this study was to assign a role for XRCC1 in the DNA processing mediated by hPNK.

DNA Binding to hPNK—An interesting difference between mammalian and T4 phage PNKS are their respective substrate preferences. Whereas the phage enzyme phosphorylates exposed 5'-OH groups more efficiently than recessed or even blunt-ended 5'-OH groups, mammalian PNK displays a preference for 5'-OH termini at SSBs or 5'-recessed termini (11, 20). The substrate preferences reflect the different roles of the two enzymes: T4 PNK is a tRNA repair enzyme (37), whereas eukaryotic PNK is a DNA repair enzyme. The crystal structures of mammalian and T4 phage PNKs have been determined (38–40). The substrate-binding site is more open in mammalian PNK than in T4 phage PNK, which could account for their respective substrate specificities. Although the crystal structure of mammalian PNK with bound nucleotide is not yet available, a three-dimensional structural model revealed that a model DNA substrate can be docked to mammalian PNK such that the double-stranded portion, the DNA backbone (positions 6–8 on the complementary strand), and the 4 nucleotides of the single-stranded 3'-overhang contact conserved positively charged residues (Arg403, Arg482, and Lys483). Thus, this model explains the minimum substrate requirements of 8 bp of double-stranded DNA (11) and the observation that at least 3 nucleotides of single-stranded 3'-overhang (20) result in optimum binding. As is evident from the Kd values (Table 2), hPNK has a higher affinity for the gapped and nicked substrates than the intact duplex, thus offering an explanation for its preferential kinase activity for internal or recessed termini. The observed difference in the binding affinity between damaged versus undamaged DNAs and double-stranded versus single-stranded oligonucleotides suggests that the enzyme is capable of differentiating between substrates and is able to target damaged sites even in the absence of accessory proteins.

The binding affinity of hPNK for its substrate at the damaged site with 5'-OH, e.g. nicked DNA (5'-OH), was higher (lower Kd) than its enzymatic product 5'-phosphodiesterase nicked DNA, but the difference was only 5-fold (i.e. 0.25 versus 1.4 μM). For an enzyme, one would expect this difference in binding affinity between substrate and product to be far greater, and hence, one could interpret that the product is not immediately released and remains bound to hPNK. However, for an enzyme to function efficiently, the generated product needs to be released, thus avoiding any possible feedback inhibition and freeing the enzyme to continue its function. In this context, we studied the possible role of the scaffolding protein XRCC1, which is known to activate PNK in addition to interacting with other proteins involved in DNA repair (13, 14, 41).

hPNK-DNA-XRCC1 Ternary Complex—Further study of the interaction of hPNK with its substrates and the scaffolding protein XRCC1 necessitated labeling of hPNK with a fluorescent probe. This was achieved by labeling with the cysteine-reactive compound AC. Labeling with AC did not appear to alter the structure or function of hPNK because the protein retained >90% of its kinase activity, and the binding affinities of hPNK-AC for its substrates determined by direct excitation of AC at 380 nm were in excellent agreement with the values...
obtained using intrinsic fluorescence. Interaction between hPNK-AC and XRCC1 was studied by monitoring the effect of XRCC1 addition on AC fluorescence at 495 nm following direct excitation of AC at 380 nm. hPNK-AC bound XRCC1 tightly in a stoichiometric manner with a $K_d$ of 30 ± 2 nM. The binding of XRCC1 to hPNK-AC was not influenced by the presence of nicked DNA (and other substrates) in terms of its binding affinity and stoichiometry. Similarly, the presence of XRCC1 had no impact on the binding of nicked DNA to hPNK-AC. These observations are consistent with formation of a ternary complex involving PNK, XRCC1, and nicked DNA.

The order of addition of XRCC1 and nicked DNA to hPNK-AC had no effect on the final emission spectrum of hPNK-AC, suggesting that XRCC1 and the substrate nicked DNA bind at different sites on hPNK-AC but sufficiently close to the labeled Cys residue so as to perturb the labeled Cys residue. The binding site of XRCC1 on hPNK has been assigned to the forkhead-associated domain at the N terminus of the protein (14, 20), and the kinase binding domain involving its DNA-binding site is believed to be at the C terminus (9, 20). The maximum quenching observed at the end of the fluorescence titrations with XRCC1 and nicked DNA was 45 and 65%, respectively. The observed quenching effects with XRCC1 and the substrate nicked DNA are likely a reflection of the distances of their respective binding sites from the labeled Cys residue.

We have demonstrated the AC-labeled Cys residue to be Cys409 based on our results obtained with the hPNK-AC mutant C409A and its interaction with model DNA ligands. Cys409 is also close to the DNA-binding site at a distance of 13 Å. The observed quenching of tryptophan fluorescence of unlabeled hPNK upon DNA binding could be attributed mainly to the perturbation of Trp402 because the model proposed for substrate binding to the kinase domain suggests that this amino acid is part of the DNA-binding site, and the edge of its indole ring interacts with the backbone phosphate (20). The labeled Cys409 residue is also close to Trp402 (11 Å) and may account for the results obtained from Trp FRET experiments in which higher quenching was obtained upon DNA binding. The forkhead-associated domain is linked to the kinase/phosphatase catalytic domain by a flexible tether (20) and hence, even though XRCC1 binding is to the forkhead-associated domain, it is still able to perturb AC fluorescence.

Roles of XRCC1 in hPNK/DNA Interaction—Binding of substrates with 5'-phosphate termini to hPNK-AC was influenced by the presence of XRCC1. Addition of substrates with 5'-phosphate termini (nicked DNA or the single-stranded 24-mer) resulted in quenching AC fluorescence, and surprisingly, the $K_d$ values obtained were only ~5-fold higher (weaker binding) compared with those of their corresponding substrates with 5'-OH. However, if XRCC1 was added first to hPNK-AC, then the substrates with 5'-phosphate were not effective in quenching AC fluorescence in contrast to substrates with 5'-OH. Because hPNK bound 5'-OH nicked DNA more tightly compared with 5'-phosphate nicked DNA ($K_d = 0.25 \mu M$), the substrates with 5'-OH were able to bind to hPNK in the presence of XRCC1 and form a ternary complex, but the presence of XRCC1 prevented the products of the reaction (nicked DNA (5'-phosphate) or the 24-mer (5'-phosphate)) from binding to hPNK. On the other hand, XRCC1 was capable of binding to the hPNK-AC-nicked DNA (5'-phosphate) binary complex. The binding affinity of nicked DNA (5'-phosphate) for XRCC1 was ~15 times tighter than that for hPNK ($K_d = 90 \mu M$ and 1.4 $\mu M$, respectively). Thus, when substrates with 5'-phosphates were added to the hPNK-AC:XRCC1 complex, XRCC1 by virtue of its high affinity for these substrates would bind them first and prevent them from binding to hPNK-AC. In the situation in which XRCC1 was added to the hPNK-AC-nicked DNA (5'-phosphate) preformed complex, it could potentially compete with hPNK-AC for nicked DNA (5'-phosphate) within a ternary complex and result in the release of nicked DNA (5'-phosphate) from hPNK-AC.

In addition to competition resulting from differential binding affinities, there is a possibility of a steric factor influencing the binding of substrates to the hPNK-XRCC1 complex. The presence of XRCC1 in the hPNK-XRCC1 complex probably exerts a steric hindrance on the binding of hPNK to 5'-phosphate nicked DNA but not to 5'-OH nicked DNA because 5'-phosphate nicked DNA was not able to bind to hPNK even when a 2-fold excess of the substrate was added. In one experiment, we added a 10-fold excess of nicked DNA (5'-phosphate) to the hPNK-AC-XRCC1 complex, and even under this condition, there was no significant quenching, suggesting that the product-binding site is not accessible. Thus, both competition and steric factors may be involved in XRCC1 modulation of PNK/substrate interactions, suggesting that 5'-OH and 5'-phosphate nicked DNAs possibly bind at different sites on PNK.

Results from lifetime measurements also suggested the release of substrates with 5'-phosphate from hPNK-AC. Addition of XRCC1 to hPNK-AC had no significant effect on the lifetime of hPNK-AC. However, upon addition of substrates with 5'-OH or 5'-phosphate to hPNK-AC, the lifetime was altered, indicating the formation of a complex in both instances; but addition of XRCC1 to the hPNK-AC-nicked DNA (5'-phosphate) binary complex or to the 24-mer (5'-phosphate) resulted in the dissociation of this binary complex because the lifetime value returned to the control value of hPNK-AC. Because addition of XRCC1 had no significant effect on the lifetime of hPNK-AC, one cannot rule out the possibility that XRCC1 remains bound to hPNK-AC with the 5'-phosphorylated product now bound to XRCC1 instead of hPNK-AC. In the case of the binary complexes involving hPNK-AC and substrates with 5'-OH (nicked DNA or the 24-mer), addition of XRCC1 resulted in a ternary complex, suggesting that these complexes are stable in the presence of XRCC1. Similarly, addition of 5'-OH substrates altered the fluorescence lifetime of hPNK-AC in the hPNK-AC-XRCC1 complex, whereas the 5'-phosphorylated products were ineffective. On the basis of these results, one may conclude that when XRCC1 is bound to hPNK, hPNK is able to differentiate between substrates with 5'-OH versus 5'-phosphate, and this may enhance its enzymatic function within the cell.

Modified Version of the Current Model for Mammalian SSB Processing—According to the model proposed for mammalian SSB repair by Whitehouse et al. (13), XRCC1, together with DNA ligase III, arrives first at the damaged site, and PNK is
subsequently sequestered for end processing by simultaneous interaction with the SSB and the XRCC1-DNA ligase III complex. Binding of XRCC1 results in the stimulation of PNK activity at the onset of SSB repair and accelerates the overall repair process. However, when looking specifically at the 3'-phosphatase activity of PNK, Parsons et al. (42) showed that binding of PNK to DNA precedes that of XRCC1, i.e. strand breaks containing modified 3'-ends are recognized first by PNK, which converts the modified 3'-ends into conventional 3’-hydroxyl ends.

On the basis of this and our own observations, we propose a modified version of the current model for mammalian SSB processing (Fig. 7). PNK initiates the repair process by binding to DNA lesion sites containing 5’-OH and converts the 5’-OH to 5’-phosphate. PNK remains bound to the 5’-phosphorylated product but perhaps in an altered conformation as evidenced by our previous Trp fluorescence quenching studies (21) and this work involving the quenching of AC fluorescence. The PNK-DNA complex containing 5’-phosphate interacts in turn with XRCC1, and during this stage, PNK is released from its phosphorylated product by XRCC1. Because XRCC1 also stimulates the phosphatase activity (13), it is likely that XRCC1 similarly displaces PNK bound to a DNA 3’-OH terminus. The results of our kinetic studies also provided evidence for the release of the product from hPNK because addition of XRCC1 resulted in the activation of hPNK by altering its rate of turnover. Our lifetime fluorescence measurements indicated that upon the arrival of XRCC1, substrates with 5’-phosphates are released from hPNK-AC. In addition, the steady-state fluorescence measurements suggested that the released product remains bound to XRCC1 for subsequent repair processing because there was no evidence for any interaction or binding between the binary complex of XRCC1 and substrates with 5’-phosphate and hPNK-AC (Table 3). Our proposed model provides a mechanism for the release of nicked DNA (5’-phosphate) from hPNK, and also, in the presence of XRCC1, hPNK is able to differentiate between nicked DNA and 5’-phosphate nicked DNA.

Although the above-mentioned scheme pertains to our in vitro studies, our results, along with the findings of Whitehouse et al. (13), could also explain events at the cellular level. XRCC1 and PNK can be co-immunoprecipitated from human cell extracts (13), and our fluorescence studies with hPNK-AC also indicate that these two proteins bind each other tightly, with a $K_d$ of 30 nM, suggesting that they coexist as a complex in cell nuclei. Our measurement of the cellular levels of the two proteins indicated a ratio of four XRCC1 molecules to every hPNK molecule (supplemental Fig. 1), further supporting the notion that a high percentage of cellular hPNK could be bound to XRCC1. XRCC1 specifically binds gapped and nicked SSB DNAs with $K_d$ values of 30 and 60 nM, respectively (29, 41); hence, it could also serve as a strand break sensor, in addition to its structural role. XRCC1 binds gapped and nicked DNAs much more tightly than PNK and thus may direct the enzyme PNK to the damaged site. In this scenario, PNK would be able to target the damaged site, and because of the presence of XRCC1, it would be able to differentiate SSBs requiring its kinase or phosphatase activities from those already possessing 5’-phosphate and 3’-OH termini.

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