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Physical and functional interaction of human nuclear uracil-DNA glycosylase with proliferating cell nuclear antigen[☆]

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Abstract

Uracil residues arise in DNA by the misincorporation of dUMP in place of dTMP during DNA replication or by the deamination of cytosine in DNA. Uracil-DNA glycosylase initiates DNA base excision repair of uracil residues by catalyzing the hydrolysis of the *N*-glycosylic bond linking the uracil base to deoxyribose. In human cells, the nuclear form of uracil-DNA glycosylase (UNG2) contains a conserved PCNA-binding motif located at the N-terminus that has been implicated experimentally in binding PCNA. Here we use purified preparations of UNG2 and PCNA to demonstrate that UNG2 physically associates with PCNA. UNG2 co-eluted with PCNA during size exclusion chromatography and bound to a PCNA affinity column. Association of UNG2 with PCNA was abolished by the addition of 100 mM NaCl, and significantly decreased in the presence of 10 mM MgCl₂. The functional significance of the UNG2-PCNA association was demonstrated by UNG2 activity assays. Addition of PCNA (30–810 pmol) to standard uracil-DNA glycosylase reactions containing linear [*uracil*-³H]DNA stimulated UNG2 catalytic activity up to 2.6-fold. UNG2 activity was also stimulated by 7.5 mM MgCl₂. The stimulatory effect of PCNA was increased by the addition of MgCl₂; however, the dependence on PCNA concentration was the same, indicating that the effects of MgCl₂ and PCNA on UNG2 activity occurred by independent mechanisms. Loading of PCNA onto the DNA substrate was required for stimulation, as the activity of UNG2 on circular DNA substrates was not affected by the addition of PCNA. Addition of replication factor C and ATP to reactions containing 90 pmol of PCNA resulted in two-fold stimulation of UNG2 activity on circular DNA.

Keywords

Human nuclear uracil-DNA glycosylase; UNG2; Proliferating cell nuclear antigen; PCNA; Functional protein interaction

1. Introduction

Uracil-DNA glycosylase initiates the DNA base excision repair pathway by excising uracil residues that arise from the deamination of cytosine or the misincorporation of dUMP in place of dTMP [1,2]. In human cells, the *UNG* gene encodes both the nuclear form (UNG2) and the mitochondrial pre-form (UNG1) of uracil-DNA glycosylase using different promoters and alternative splicing [3]. UNG1 and UNG2 have unique N-terminal domains

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required for subcellular sorting, while the core catalytic domain (UNG Δ 84) is common to the two forms [3,4]. All forms of UNG examined remove uracil opposite A or G in double-stranded DNA as well as from single-stranded DNA [3,5]. At least four different human nuclear DNA glycosylases, UNG2, single-strand selective monofunctional uracil-DNA glycosylase (SMUG1), thymine-DNA glycosylase (TDG) and methyl-CpG binding domain protein 4 (MBD4, also known as MED1) have been reported to removal uracil from U-G mispairs [6–8], and it has not yet been determined whether the different uracil-DNA glycosylases have distinct or overlapping physiological functions. However, the recent experiments by Akbari and co-workers indicate that UNG2 is the major activity to initiate base excision repair of deaminated cytosine (U-G) and perhaps the only enzyme to remove misincorporated uracil (U-A) [6]. A role for UNG2 in somatic hypermutation and class-switch recombination has been implicated (for reviews, see [9–11], and recently it was reported that UNG2 was recruited into HIV particles by HIV integrase and played an essential role in the viral life cycle [12].

Proliferating cell nuclear antigen (PCNA) was originally identified by Takasaki et al. [13] as a nuclear antigen associated with cell proliferation that was recognized by autoantibodies in the sera of some patients with systemic lupus erythematosus. Subsequently, PCNA was identified by Stillman and co-workers as a DNA elongation factor for SV40 DNA replication in vitro that increased the processivity of calf thymus DNA polymerase δ [14,15]. PCNA is a highly conserved eukaryotic homotrimeric protein that assembles around DNA to form a sliding clamp and acts as a processivity factor for the replicative polymerase (reviewed in [16]). PCNA interacts with a large variety of proteins involved in DNA replication, lesion bypass, DNA repair, and cell cycle control, such as DNA polymerases δ and ϵ [17], DNA polymerase η [18], LIG1 [19], MSH3 and MSH6 [20], FEN1 [21], GADD45 [22], and p21 [23]. An eight amino acid conserved motif (Q-x-x-[I/L/M]-x-x-F-[F/Y]) in these proteins modulates their binding to PCNA [24]. In general, the motif is found either at the N- or the C-terminus, frequently at the extreme terminus of the polypeptide. The crystal structure of human PCNA in complex with a p21 peptide showed extensive interactions through the formation of a β -sheet between the peptide and the interdomain connection loop (IDCL) of PCNA [25]. Ile126 and Leu128 in the IDCL were part of a hydrophobic cavity into which Met4 and Tyr8 of the p21 motif were inserted. Mutations in the hydrophobic core of the IDCL of PCNA abolished p21 binding [26].

In UNG2, the putative PCNA-binding motif, **QKTLYSFF** (highly conserved residues indicated in bold), is present at amino acids 4–11 of the 313 amino acid polypeptide. Otterlei et al. [4] demonstrated that PCNA co-localized with Replication Protein A and UNG2 in replication foci, and a molecular interaction of UNG2 with PCNA was demonstrated using two-hybrid assays. Recently, PCNA-binding proteins from human cell lysates were identified by a combination of affinity chromatography and mass spectrometric analyses, and UNG2 was identified among the more than 20 proteins that bound to a PCNA affinity column [27]. In the present study, we have examined the physical and functional aspects of the UNG2-PCNA interaction using purified proteins. We demonstrate that UNG2 physically interacts with PCNA in vitro and that PCNA stimulates the uracil excision activity of UNG2. The significance of the findings is discussed with respect to the physiological role of the UNG2 N-terminal domain.

2. Materials and methods

2.1. Materials

pET-28a-UNG2 was provided by Dr. Geir Slupphaug (Norwegian University of Science and Technology), pQE30hPCNAwt by Dr. Paul Fisher (State University of New York, Stony Brook), and pET-28A-LC59A (PCNA L126D/I128E) by Dr. Ronald Gary (University of

Nevada, Las Vegas). Purified replication factor C (RFC), in which the N-terminal 555 amino acids of the p140 subunit were deleted [28], was provided by Dr. J. Hurwitz (Memorial Sloan-Kettering Cancer Institute). BL21(DE3) was purchased from Stratagene Corporation, as was pRIL. Hydroxyapatite Bio-HTP Gel and CM Macro-prep were from Bio-Rad; Sephacryl S-300 HR was from Amersham Bioscience.

2.2. Preparation of DNA substrates

Calf thymus [*uracil*-³H]DNA (175 cpm/pmol of uracil) was prepared in a nick-translation reaction using activated DNA and *E. coli* Pol I as described by Mosbaugh and Linn [29]. M13mp2opl4 DNA was propagated in *E. coli* JM109 cells as described by Sanderson and Mosbaugh [30], and single-stranded M13mp2op14 DNA was purified from the culture supernatant using the CTAB DNA precipitation method [31,32]. The single-stranded M13 DNA circles were annealed to a complementary 5'-phosphorylated oligonucleotide (5'-CCCAGTCACGTCATTGTAAAACG-3') in reactions (6 μ l $\times$ 50 μ l) containing 20 mM Tris-HCl pH 7.4, 2 mM MgCl₂, 50 mM NaCl, 0.4 μ M ssM13, and 1.2 μ M 5'-end phosphorylated oligonucleotide. The mixtures were incubated 85, 75, 70, 65, 60, 55, 50, 45, 35, 25, 15, and 5 °C for 2 min at each temperature. A portion of the annealing reaction was used in a primer extension reaction (1.6 ml) that consisted of 20 mM Hepes-KOH (pH 7.8), 2 mM DTT, 10 mM MgCl₂, 10 mM ATP, 500 μ M each of dATP, dCTP, and dGTP, 425 μ M dTTP, 50 μ M dUTP, 25 μ M [³H]dUTP, 63 μ M primed single-stranded M13 DNA circles, 150 units of T4 DNA polymerase purified as described by Sanderson and Mosbaugh [30], and 360 units (Weiss) of T4 DNA ligase (Fermentas). The primer extension reaction was incubated at 0 °C for 5 min, 22 °C for 5 min, and 37 °C for 3 h, and was terminated by the addition of EDTA to 10 mM. Form I and Form II M13 [*uracil*-³H] DNA were isolated by cesium-chloride ethidium bromide density centrifugation as described by Bennett et al. [33]. Form III M13 [*uracil*-³H] DNA (linear DNA) was prepared from Form II M13 [*uracil*-³H] DNA by *Eco*RI restriction endonuclease digestion. The specific ³H radioactivity of the M13 DNA preparations was 560 cpm/pmol of uracil.

2.3. Protein purification

The nuclear form of human uracil-DNA glycosylase (UNG2) was produced and purified as follows. Rich broth (2 $\times$ YT, 6 l) containing 25 μ g/ml kanamycin, 34 μ g/ml chloramphenicol, and 0.67% glucose was inoculated (1/100) with an overnight culture of BL21(DE3)/pRTL/pET-28a-UNG2 grown in the same medium, and incubated at 37 °C with shaking until the optical density (*A*₆₀₀) reached 0.5. The incubation temperature was then lowered to 27 °C and, after 30 min, IPTG was added to a final concentration of 1 mM to induce overproduction of his₆-tagged UNG2. After 5 h cells were harvested by centrifugation, suspended in 180 ml of buffer A (50 mM NaPO₄ pH 8.0, 1.0 M NaCl, 5% glycerol, 2 mM β -mercaptoethanol, 10 mM NaHSO₃ pH 7.0, 10 mM imidazole and 2 mM benzamidine) supplemented with 5 μ g/ml leupeptin, 5 μ g/ml aprotinin and 1 tablet/40 ml of Complete® (Roche) protease inhibitor, and stored at -80 °C. The cell suspension was thawed in ice water, sonified, and clarified by centrifugation. The supernatant was loaded onto an Ni-NTA (QIAGEN) column (5.3 cm² $\times$ 5.6 cm) equilibrated with buffer A. The column was washed with 200 ml of the same buffer, and the protein was eluted with buffer A containing 300 mM imidazole and 300 mM NaCl. Fractions enriched for his₆-tagged UNG2 were pooled, dialyzed against HA buffer (10 mM KPO₄ pH 8.0, 20 mM NaCl, 5% glycerol, 10 mM NaHSch, 1 mM benzamidine), and loaded onto a hydroxyapatite column (4.9 cm² $\times$ 6.5 cm) equilibrated in HA buffer. The column was washed with 160 ml of the HA buffer and eluted with a 300 ml gradient from 10 to 300 mM KPO₄ (pH 8.0) in HA buffer. Fractions containing UNG2 were pooled, dialyzed against PU buffer (30 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 5% glycerol, 10 mM NaHSO₃, 1 mM benzamidine), and applied to a poly U Sepharose column (2.01 cm² $\times$ 8 cm) equilibrated in

PU buffer. The column was washed with 110 ml of PU buffer and eluted with a 160 ml gradient from 0 to 1 M NaCl in PU buffer. Fractions containing his₆-tagged UNG2 were pooled and dialyzed against TNG buffer (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5% glycerol). The N-terminal his₆-tag (17 amino acids) was removed in a thrombin cleavage reaction (1.0 ml) that contained 185 µg of his₆-UNG2 and 18 units of thrombin in TNG buffer supplemented with 2.5 mM CaCl₂. The reaction mixture was incubated at 25 °C for 1 h, terminated by the addition of 22 µl of 0.5 M EDTA and 11 µl of 100 mM benzamidine, dialyzed against CM buffer (30 mM Tris-HCl pH 7.5, 10 mM EDTA, 1 mM DTT, 1 mM benzamidine, 10 mM NaHSO₃, 5% glycerol), and loaded onto a CM Macro-prep column (2.01 cm² × 6.5 cm). The column was washed with 65 ml of CM buffer and eluted with a 150 ml gradient from 0 to 1 M NaCl in CM buffer. Fractions containing UNG2 were pooled, concentrated in a Centricon 30 (Millipore) and buffer exchanged into DA buffer (Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol). Following thrombin cleavage, the UNG2 protein construct had three additional amino acids (Gly, Ser, His) at the N-terminus. The concentration of UNG2 was determined by UV absorbance spectroscopy using the molar extinction coefficient $\epsilon_{280\text{ nm}} = 50.4 \times 10^3 \text{ l/mol cm}$.

His₆-PCNA was overproduced in *E. coli* BL21/pQE30hPCNAwt (312× YT broth) at 37 °C by induction at early log phase growth with 1 mM IPTG. After 3 h, cells were collected by centrifugation, resuspended in buffer A (60 ml), and stored at -80 °C. The cell suspension was thawed in ice water, sonified, and clarified by centrifugation. The supernatant was loaded onto an Ni-NTA agarose column (2.01 cm² × 7 cm), which was washed with 70 ml buffer A and eluted with 100 ml gradient from 20 to 300 mM imidazole in buffer A that contained 300 mM NaCl. The fractions containing his₆-PCNA were pooled, dialyzed in HA buffer containing 2 mM DTT, and loaded onto a hydroxyapatite column (4.9 cm² × 5.7 cm). The column was washed with 56 ml HA buffer with 2 mM DTT and eluted with a 300 ml gradient of 10–600 mM KPO₄ (pH 8.0) in HA buffer. The fractions containing his₆-PCNA were pooled and dialyzed against storage buffer (25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM DTT, 10% glycerol, 20% sucrose, 0.02% NP-40). The concentration of the his₆-PCNA preparation was determined by the Bradford reaction [34] using the Protein Assay Reagent (Bio-Rad).

The core catalytic domain of UNG (UNG Δ 84), which lacks the N-terminal 84 amino acids of UNG2 [5], was overproduced in *E. coli* and purified as described by Chen et al. [35]. PCNA LC59 was overproduced in *E. coli* BL21 (DE3) and purified as described previously for wild-type PCNA, except that the LC59 double mutant flowed through, rather than binding to, the phenyl-Sepharose column [32].

2.4. Uracil-DNA glycosylase activity assays

Standard uracil glycosylase reaction mixtures (100 µl) contained buffer H (70 mM Hepes-KOH (pH 7.9), 1 mM EDTA, 1 mM DTT), 2.75 µg of calf thymus [*uracil*-³H] DNA (175 cpm per pmol uracil), and 0.09 fmol of UNG2, and were incubated at 37 °C for 30 min. The reactions were terminated by the addition of 250 µl of 10 mM ammonium formate (pH 4.2), and free [³H] uracil was resolved from calf thymus [*uracil*-³H] DNA by mixed bed ion exchange chromatography and quantified using a Beckman 6500 liquid scintillation counter as described by Bennett and Mosbaugh [36]. One unit of uracil-DNA glycosylase was defined as the amount that releases 1 nmol of uracil/h under standard conditions.

To assess the effect of PCNA on UNG2 activity, uracil-DNA glycosylase reactions (100 µl) were conducted that contained 2.6 µg of M13 [*uracil*-³H]DNA, either Form I or Form III, buffer H, and varying amounts (0–810 pmol) of PCNA. Following incubation at 37 °C for 30 min, the reactions were terminated and excised ³H uracil quantified as described above. To determine the effect of RFC on UNG2 activity in reactions that contained PCNA,

reaction mixtures were prepared that contained 25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM DTT, 7.5 mM MgCl₂, 2 mM ATP, 2.6 µg of M13[uracil-³H]DNA, either Form I or Form III, 6 fmol of UNG2, 90 pmol of PCNA, and either 0 or 2 µg of RFC. Reactions were incubated at 37 °C for 30 min, and then terminated with ammonium formate; the amount of ³H uracil was quantified by liquid scintillation counting as described.

2.5. Ni-NTA agarose affinity chromatography

Ni-NTA agarose was equilibrated in TNT buffer (25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM DTT) and a 50% slurry (250 µl) was transferred to a mini spin column (Bio-Rad), which was allowed to drain by gravity. Mixtures (50 µl) of various amounts (0–600 pmol) of UNG2 and his-tagged PCNA were prepared in TNT buffer containing 20 mM imidazole and 10% glycerol and incubated at 25 °C for 10 min prior to column loading. Unbound proteins were removed by washing the column five times with 250 µl TNT buffer that contained 60 mM imidazole-HCl (pH 7.5). The Ni-NTA agarose matrix was then collected in a 2 ml microcentrifuge tube and bound proteins were eluted by the addition of 100 µl of 2× SDS sample buffer [37] and boiling for 10 min. Following the centrifugation at 3000 × g, the supernatant was removed and portions (20 µl) were analyzed by 12.5% SDS-PAGE and Coomassie Brilliant Blue G-250 staining to determine the amount of UNG2 bound to the PCNA. The intensity of the stained protein bands was quantified using a ChemiGenius^{2XE} Bio Imaging System (Syngene) and compared to UNG2 and PCNA protein standards on reference gels.

2.6. Analysis of the UNG2–PCNA interaction by size exclusion chromatography

A Sephacryl S-300 HR size exclusion column (0.64 cm² × 55 cm) was equilibrated with TNT buffer. Reaction mixtures (50 µl) containing PCNA, UNG2, or Δ84 were incubated in TNT buffer with 10% glycerol on ice for 10 min prior to column loading. The column was then eluted with TNT buffer at a flow rate of 30 ml/h and fractions (470 µl) were collected. To determine the protein concentration of the fractions, samples (200 µl) were transferred to a micro titer plate and mixed with 50 µl of Protein Assay Reagent (Bio-Rad) and the absorbance at 595 nm was determined using a Spectra MAX 250 (Molecular Devices) and compared to the absorbance of UNG2 and PCNA protein standards. The elution profile of UNG2 was determined using standard uracil-DNA glycosylase assays. The elution profile of PCNA was also determined by immunodot blot analysis. Portions (200 µl) of the elution fractions were applied to a Protran nitrocellulose membrane (Schleicher & Schuell) installed in a Minifold I custom modified vacuum manifold [38] and equilibrated in TBS buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl). Each well of the manifold was washed with 500 µl of TBS buffer and the membrane was removed and blocked for 20 min (Superblock, Pierce Chemical Co.) The membrane was then incubated with mouse anti-hPCNA monoclonal antibody (Abcam) diluted 1–8000 in dilution buffer (20 ml TBS with 10% Superblock and 0.05% Tween-20) for 1 h and subsequently washed with dilution buffer five times. Next, the membrane was incubated with goat anti-mouse IgG (Novus Biologicals) conjugated to horseradish peroxidase (1/80,000 dilution), washed, and developed with Super-Signal West Pico chemiluminescence substrate (Pierce). Chemiluminescence was quantified using the ChemiGenius^{2XE} Bio Imaging System (Syngene).

3. Results

3.1. Purification of UNG2 and PCNA

Since the N-terminus of UNG2 was reported to be susceptible to proteolysis [39], UNG2 was produced in *E. coli* as an N-terminal his₆-tag fusion protein. Following purification to apparent homogeneity (Fig. 1A), the his₆-tag sequence was cleaved with thrombin to generate UNG2 with an intact N-terminus that contained three additional amino acids (Gly,

Ser, His). Reports from the laboratories of Wang [6] and Fisher [40] indicated that the introduction of a his-tag at the amino terminus of PCNA did not affect PCNA homotrimer formation or stimulation of DNA polymerase δ synthetic activity; both laboratories overproduced his-tagged PCNA in *E. coli*. Therefore, we utilized the construct provided by Fisher and co-workers [40] to overproduce human his₆-tagged PCNA in *E. coli* and purified the recombinant protein to apparent homogeneity as shown in Fig. 1B. When subjected to Sephacryl S-300 size exclusion chromatography, the purified his₆-tagged PCNA preparation eluted as a ~85 kDa homotrimer, indicative of wild-type functionality (Section 3.4). The PCNA double mutant, LC59A, was produced without an affinity tag and purified using the method of Fien and Stillman [32] with one modification. While wild-type PCNA bound to phenyl-Sepharose at 1.2 M NaCl, PCNA LC59 flowed through. This may be due to the double mutation (L126D/I128E) in the protein, where two hydrophobic amino acid residues in the solvent-exposed interdomain connecting loop were changed to acidic residues.

3.2. Effect of metal ions on UNG2 activity

Since Kavli et al. [39] reported that MgCl₂ stimulated UNG2 activity, we were curious to learn about the effects of other divalent metal cations on UNG2 activity. Therefore, in addition to MgCl₂, MnCl₂, FeSO₄, CoCl₂, NiSO₄, CuSO₄, and ZnSO₄ (7.5 mM each) were added to standard uracil glycosylase activity assays containing either UNG2 or UNG δ 84 and nick translated calf thymus [*uracil*-³H]DNA. Compared to the control reaction conducted in the absence of metal ions, UNG2 enzyme activity increased two-fold in the presence of 7.5 mM MgCl₂ (Fig. 2). When the standard assay was supplemented with 7.5 mM MnCl₂, a 1.8-fold stimulation of activity was observed. In contrast, the same concentration of FeSO₄, CoCl₂, NiSO₄, CuSO₄, or ZnSO₄, was inhibitory to UNG2 activity (Fig. 2). In fact, inhibition by Ni²⁺, Cu²⁺, and Zn²⁺ was almost complete: virtually no activity was detected. The activity of UNG δ 84 was inhibited by all the metal ions tested. Thus, stimulation by Mg²⁺ (and Mn²⁺) was specific for UNG that contained the full length N-terminal domain, as noted by Kavli et al. [39]. The mechanism by which these ions stimulate or inhibit uracil-DNA glycosylase activity warrants further investigation.

3.3. UNG2 binding to immobilized PCNA

Using short peptides from the UNG2 N-terminal region, Otterlei et al. [4] demonstrated that a biotinylated peptide corresponding to the first 20 amino acids of UNG2 bound to PCNA in a streptavidin-agarose bead pull-down experiment. To determine whether the full-length UNG2 protein could physically interact with homotrimeric PCNA, a binding assay was conducted using a Ni-NTA agarose his₆-affinity column (Fig. 3). In order to facilitate quantitative detection of UNG2 and PCNA in Coomassie-stained SDS-polyacrylamide gels, a standard curve was constructed as follows (Fig. 3A). Mixtures of UNG2 and PCNA in various molar ratios were prepared and resolved by SDS-PAGE. After staining with Coomassie Brilliant Blue G250, the gels were destained and the intensities of the UNG2 and PCNA protein bands were quantified using a digital imaging system as described under Section 2. The stoichiometry of the mixtures was then graphed as a function of the ratio of the UNG2/PCNA band intensities. In this fashion the stoichiometry of UNG2 and PCNA in an SDS-polyacrylamide gel could be determined by the ratio of the stained protein bands in the gel lane. The standard curve shows that the affinity of the UNG2 protein for Coomassie Brilliant Blue G250 was 1.5-fold greater than PCNA (Fig. 3A). Next, various amounts of purified UNG2 were incubated with his₆-tagged PCNA and applied to the Ni-NTA agarose column as described under Section 2. In order to avoid non-specific binding of UNG2 to the column matrix, the wash buffer was adjusted to 60 mM imidazole. After extensive washing, the columns were eluted with SDS sample buffer and the eluates analyzed by SDS-PAGE and Coomassie staining. The intensity of the UNG2 and PCNA stained protein bands was quantified, and the stoichiometry of the UNG2 and PCNA proteins recovered was

determined using the UNG2/PCNA standard curve shown in Fig. 3A. A binding curve was obtained by titration of 200 pmol of PCNA (monomer) with 0–600 pmol of UNG2, and appeared to saturate at ≥ 200 pmol of UNG2 (Fig. 3B). The amount of UNG2 bound to PCNA at saturation was about 133 pmol, or 67% of the amount of PCNA detected. This result suggests that perhaps two molecules of UNG2 on average bound to a PCNA homotrimer under these conditions. However, the effects of the Ni-NTA agarose matrix and the assay conditions on the UNG2/his₆-tagged-PCNA interaction are not known, and a more precise determination of the stoichiometry of the UNG2/PCNA interaction must await further experimentation.

To examine the effect of NaCl on the UNG2–PCNA interaction, various concentrations of NaCl (0–200 mM) were included in the column equilibration buffer, sample incubation buffer and wash buffer, and the Ni-NTA agarose binding assay was performed (Fig. 3C). The results show that UNG2 binding to PCNA decreased in the presence of NaCl. Relative to the binding observed in the absence of NaCl, the amount of UNG2 bound to PCNA decreased 29% at 25 mM NaCl, and >95% at 100 mM NaCl (Fig. 3C). The effect of MgCl₂ on the UNG2–PCNA interaction was also determined (Fig. 3D). MgCl₂ (0–10 mM) was added to the column equilibration buffer, sample incubation buffer, and wash buffer, and the binding assay performed as described above. Relative to UNG2 binding in the absence of MgCl₂, binding of UNG2 to PCNA decreased 70% at 2.5 mM MgCl₂ and >90% at 10 mM MgCl₂ (Fig. 3D).

3.4. Size exclusion chromatography of the UNG2–PCNA complex

To further investigate the interaction of UNG2 with PCNA, size exclusion chromatography of PCNA, UNG2, and a mixture of PCNA and UNG2 was performed using Sephacryl S-300 HR (Amersham Biosciences). The column void volume occurred at fraction 24, whereas the protein size standards, bacterial alkaline phosphatase (80–89 kDa) [41,42] and carbonic anhydrase (29 kDa) eluted at fractions 34 and 41, respectively. When the his₆-tagged PCNA preparation was loaded on the column, it eluted as a symmetrical peak centered at fraction 34 (peak I in Fig. 4A), which corresponded to a molecular weight of approximately 85 kDa, indicating that the his₆-tagged PCNA migrated as a trimer [43]. The elution profile of PCNA was confirmed by immunodot blot analysis using an anti-hPCNA mouse monoclonal antibody. When UNG2 was applied to the column, a symmetrical protein peak was detected that was centered on fraction 43, which also corresponded to the peak of uracil-DNA glycosylase activity (peak II in Fig. 4B). When a mixture of UNG2 (250 pmol) and PCNA (1 nmol) were applied to the column, the peak of uracil-DNA glycosylase activity shifted from fraction 43 to fraction 34 (peaks I + II in Fig. 4C), suggesting that a portion of UNG2 co-migrated with PCNA during elution from the column; approximately 70% of total UNG2 activity was detected in fraction 34. In contrast, when 100 mM NaCl was included in the sample and column buffer, two separate protein elution peaks were observed at fraction 34 (peak I) and fraction 43 (peak II), and all the UNG2 activity shifted to peak II (Fig. 4D). In a control experiment, PCNA was combined with UNG Δ 84, the UNG2 core catalytic domain lacking the first 84 N-terminal amino acids. As expected, two separate protein peaks were observed at fraction No. 34 (peak I) and No. 49 (peak III); the peak of uracil-DNA glycosylase activity corresponded to peak III (Fig. 4E). These results confirmed that the N-terminal region of UNG2 was required for the UNG2–PCNA interaction.

3.5. Effect of PCNA on UNG2 catalytic activity

To determine whether the physical interaction of PCNA with UNG2 had functional consequences for UNG2 catalytic activity, we conducted standard uracil-DNA glycosylase reactions to which 0–1620 pmol of purified PCNA was added (Fig. 5A). In a control experiment, PCNA was also added to reactions containing UNG Δ 84. The results show that

the activity of UNG2 increased gradually as the concentration of PCNA increased. UNG2 activity was stimulated 2.6-fold by the addition of 1620 pmol of PCNA, whereas no stimulation of uracil-DNA glycosylase activity was observed in reactions containing UNG Δ 84 (Fig. 5A). Since MgCl₂ was also observed to stimulate UNG2 activity (Fig. 2), we wondered whether the combination of MgCl₂ and PCNA would have a synergistic effect on UNG2 activity. Therefore, standard uracil-DNA glycosylase reactions were conducted in the absence and presence of 7.5 mM MgCl₂ and increasing amounts of PCNA (Fig. 5B). The effect of PCNA on UNG2 activity was similar in both reactions; however, the PCNA-mediated stimulation of UNG2 activity was approximately two-fold higher in the presence of MgCl₂ at all PCNA concentrations tested (Fig. 5B). This observation led us to conclude that the stimulatory effects of MgCl₂ and PCNA occurred by independent mechanisms.

3.6. Influence of mutations in the interdomain connector loop of PCNA on the UNG2–PCNA interaction

The extreme N-terminus of UNG2 bears a consensus PCNA-binding motif shared by p21, MSH6, LIG1, XPG, FEN1, and many other proteins involved in DNA transactions [44]. In a seminal crystal structure of PCNA, a p21 peptide encompassing the binding motif was shown to bind to the interdomain connector loop (IDCL) region of PCNA [25]. Flap endonuclease 1 (FEN1) interacts with PCNA by a mechanism analogous to that of p21 [45], and a PCNA IDCL double point mutant, L126D/I128E (called LC59A), was shown to be severely defective in FEN-1-binding [46]. Therefore, to investigate the possible involvement of the PCNA IDCL in the UNG2–PCNA interaction, PCNA LC59A was overproduced in *E. coli*, purified, and tested for interaction with UNG2 and stimulation of UNG2 catalytic activity (Fig. 6). If the interaction of UNG2 with PCNA was analogous to that of FEN1, then one would predict that PCNA LC59A would be defective in interacting with UNG2. To test this hypothesis, PCNA LC59A and UNG2 were combined and applied to the Sephacryl S-300 size exclusion column; the elution profiles of the two proteins are shown in Fig. 6A. The peak of PCNA LC59A elution occurred at fraction 33, indicating that the IDCL mutant PCNA formed a homotrimer, consistent with the report by Gary et al. [46] that the mutant PCNA formed an 87 kDa homotrimer. The peak of UNG2 activity shifted from fraction 43 (demonstrated in Fig. 4B) to fraction 33, the peak of PCNA LC59A elution. Approximately 70% of UNG2 activity resided in the PCNA LC59A elution peak, as was found previously for wild-type PCNA (Fig. 4C). This result indicated that the L126D/I128E double point mutation did not appear to affect co-migration of UNG2 with PCNA LC59A during size exclusion chromatography. We then examined the ability of PCNA LC59A to stimulate UNG2 catalytic activity in standard uracil-DNA glycosylase reactions (Fig. 6B). Compared to the stimulatory effect of wild-type PCNA at 1620 pmol (~3.3-fold), the maximum stimulatory effect of the LC59A mutant (~1.9-fold) was 58% lower than wild-type PCNA. In addition, the amount of PCNA LC59A required for maximal stimulation of UNG2 activity (~100 pmol) was much less than the amount of PCNA (870 pmol) required (Fig. 6B). The cause of this decline in the stimulatory effect of PCNA LC59A is not clear, but may be the result of altered endloading of PCNA LC59A onto the [uracil-³H]DNA substrate. Alternatively, mutations in the IDCL of PCNA may cause a subtle change in the mode of interaction with UNG2.

3.7. Effect of RFC on PCNA-mediated stimulation of UNG2 activity

The observations that UNG2 interacted with PCNA in the absence of DNA (Figs. 3 and 4), and that addition of PCNA to standard uracil-DNA glycosylase reactions resulted in a stimulation of UNG2 activity (Fig. 5), led us to consider whether loading of PCNA onto DNA was required for stimulation. Burgers and Yoder [47] reported that PCNA could stimulate synthesis by DNA polymerase δ on linear templates by slipping onto the end of the DNA in an ATP-independent manner, although a large molar excess was required for

maximal stimulation. Since large amounts (810 pmol) of PCNA were required in order to detect stimulation of UNG2 activity, was passive end-loading of PCNA onto the calf thymus [*uracil*-³H]DNA substrate responsible for the stimulatory effect? To investigate this hypothesis, we constructed a closed circular (Form I) M13 [*uracil*-³H]DNA substrate. We reasoned that since PCNA cannot load itself onto circular DNA [28], PCNA-mediated stimulation of UNG2 activity would occur only if the protein:protein interaction of UNG2 with PCNA was the source of stimulation. However, if loading of PCNA onto the [*uracil*-³H]DNA substrate was required for the stimulatory effect, no stimulation of UNG2 activity would be observed using the Form I substrate. A control linear substrate was produced from nicked circular (Form II) M13 [*uracil*-³H]DNA by restriction with *Eco*RI, which created a 7196 bp linear (Form III) M13 [*uracil*-³H]DNA molecule. The Form I and Form III M13 [*uracil*-³H]DNA substrates were reacted in the absence or presence of PCNA (810 pmol), and the amount of excised ³H uracil was quantified as described under Section 2; the results are shown in Fig. 7A. When Form I DNA was used as substrate for UNG2, the large molar excess of PCNA has no effect on UNG2 activity compared to the same reaction without PCNA. In contrast, addition of the same amount of PCNA to reactions containing Form III DNA resulted in a two-fold stimulation of UNG2 activity (Fig. 7A). Since PCNA cannot end load onto Form I DNA, these results indicated that (1) the physical interaction of UNG2 with PCNA alone was not sufficient for stimulation of UNG2 activity, and (2) that the PCNA-mediated stimulatory effect on UNG2 activity using Form III DNA was most likely the result of PCNA passive end-loading onto the 7,196 bp linear DNA substrate. The large excess of PCNA was required to observe stimulation of UNG2 activity since PCNA slides off the ends of linear DNA [43]. Interestingly, in the absence of PCNA, UNG2 was about 2.5-fold more active on the Form I DNA substrate compared to the identical, but linear, Form III DNA substrate (Fig. 7A). While the mechanism underlying the increased activity of UNG2 on circular compared to linear DNA is not clear, it may be possible that UNG2 dissociates more quickly from linear DNA than from circular DNA.

To further investigate the mechanism of the PCNA stimulatory effect on UNG2 activity, replication factor C (RFC), as well as ATP and Mg²⁺, were included in UNG2 activity reactions (Fig. 7B). In the presence of ATP and Mg²⁺, the five-subunit RFC protein binds PCNA and, concomitant with ATP hydrolysis, assembles the PCNA trimer around the DNA [43]. RFC also functions as a clamp unloader, dissociating PCNA from circular DNA in an ATP-hydrolysis-dependent process [43]. When circular DNA containing bound PCNA was separated from RFC, the half-life of PCNA dissociation from the DNA was reported to be 24 min [43]. However, with PCNA and RFC present in the UNG2 activity reactions, the amount of PCNA loaded onto the Form I DNA substrate would be determined by the ATP-dependent equilibrium between loaded and free PCNA. In order to determine unambiguously whether RFC would affect the PCNA-mediated stimulation of UNG2 activity, we added a relatively small amount of PCNA (90 pmol) to the reactions that contained either Form I M13 [*uracil*-³H]DNA, Form III M13 [*uracil*-³H]DNA, or nick-translated calf thymus [*uracil*-³H]DNA (Fig. 7B). The results show that UNG2 activity increased more than two-fold in reactions with Form I M13 [*uracil*-³H]DNA, RFC, ATP, and PCNA, compared to the reaction lacking RFC. Interestingly, a similar result was observed in reactions containing Form III M13 [*uracil*-³H]DNA and the complement of PCNA, RFC, and ATP (Fig. 7B). In this case, inclusion of RFC, ATP, and Mg²⁺ together with only 90 pmol of PCNA resulted in the same two-fold level of stimulation observed for 810 pmol of PCNA in the absence of RFC (Fig. 7A). When nick-translated calf thymus [*uracil*-³H]DNA was reacted with UNG2 in the presence of RFC, ATP, and PCNA, UNG2 activity was stimulated about 35% relative to RFC control (Fig. 7B). This level of RFC-mediated stimulation was significantly less than that observed for the Form III M13 DNA substrate. We speculate that the length of the linear DNA substrate may have played a role in the degree of RFC-mediated stimulation observed, since the average length of the calf

thymus DNA substrate was about 1800 bp compared to the 7196 bp Form III M13 DNA substrate. RFC-loaded PCNA might slip off the ends of the shorter calf thymus DNA substrate more rapidly compared to the Form III M13 DNA substrate; thus, the equilibrium concentration of DNA-bound PCNA would be lower for the calf thymus DNA substrate. Lastly, in the control experiment, stimulation of UNG2 activity by RFC alone was marginal (15%) (Fig. 7B). These experiments demonstrated that RFC-mediated loading of PCNA onto DNA resulted in stimulation of UNG2 activity and suggested that PCNA may act as a tether to reduce UNG2 dissociation from substrate DNA.

4. Discussion

In this report we have demonstrated that purified UNG2 and PCNA interact physically and functionally. Experiments utilizing Ni-NTA agarose affinity chromatography and size exclusion chromatography showed that UNG2 and PCNA interacted physically, whereas addition of PCNA to UNG2 activity assays resulted in stimulation of UNG2 catalytic activity. Although a physical interaction between UNG2 and PCNA was suggested by the peptide pull-down experiments of Otterlei et al. [4] and the proteomics approach of Ohta et al. [27], the results presented here provide the first evidence of the physical interaction using purified proteins and demonstrate for the first time the functional significance of the interaction.

We observed that when ≥ 200 pmol of UNG2 were combined with 200 pmol of PCNA and applied to an Ni-NTA agarose column, the amount of UNG2 recovered was about 67% of the amount of PCNA recovered, suggesting that perhaps two molecules of UNG2 were bound per homotrimeric PCNA molecule under the conditions of the assay. A similar observation regarding the p21-PCNA interaction was reported by Flores-Rozas et al. [48], who found that on average 2.3 molecules of p21/Cipl (monomer) bound to 1 molecule of PCNA trimer. The N-terminus of UNG2 shares the PCNA consensus binding motif with several proteins involved in DNA replication and repair, including MSH3, MSH6, FEN1, MCM1, and LIG1 [44]. In light of these many interactions, it is attractive to envision a model in which UNG2 participates in a multi-protein complex associated with the replication fork in order to remove uracil residues that result from dUMP incorporation in place of dTMP opposite dAMP. However, we observed that $>95\%$ of the UNG2-PCNA complex was abolished by the addition of 100 mM NaCl to the binding; thus, the interaction of UNG2 with PCNA was not as strong as that of other proteins. For example, Unk et al. [49] reported that the interaction of yApn2 protein with PCNA was stable in 300 mM NaCl. Additional factors may be required to stabilize the UNG2-PCNA interaction in the cell.

An important question concerns how the UNG2-PCNA interaction contributes to the role of UNG in the removal of uracil-DNA damage. One possibility is that PCNA that is loaded onto DNA may increase the processivity of UNG2 by decreasing the dissociation of the enzyme from DNA. Einolf and Guengerich [50] found that the K_d of calf thymus DNA polymerase δ for DNA increased five-fold in the absence of PCNA. The authors concluded that PCNA appeared to affect Pol δ replication mainly by decreasing the dissociation of the polymerase from DNA [50]. A second possibility is that the UNG2-PCNA interaction serves to increase the affinity of UNG2 for (non-uracil-containing) DNA, which is relatively low. In this regard, Bennett et al. determined the K_d of *E. coli* Ung for single-stranded M13 DNA (no uracil) to be 600 μ M [51]. Experiments presented here (Fig. 7A) demonstrated that PCNA must be loaded onto DNA in order to stimulate UNG2 activity; in other words, the physical interaction of UNG and PCNA in solution did not stimulate UNG2 activity. We observed that large amounts of PCNA (up to 1620 pmol) were required to stimulate UNG2 on linear DNA. We interpret this observation to indicate that PCNA easily dissociated from the linear DNA by slipping off the ends. However, when PCNA was loaded by RFC onto

circular [*uracil*-³H]DNA, just 90 pmol of PCNA provided the same degree of UNG2 stimulation (Fig. 7B).

Several studies indicated that proteins containing the PCNA-binding motif bound to the interdomain connecting loop (IDCL) of PCNA by a mechanism analogous to that of p21 [46,52,53]. In order to investigate the role of PCNA interdomain connecting loop in the UNG2-PCNA interaction, we tested the ability of the LC59A PCNA mutant, which contains two mutations (I126D/L128E) in the IDCL, to bind UNG2 (Fig. 6). Gary et al. [46] reported that while wild-type PCNA bound avidly to FEN-1, the PCNA I126D/L128E mutation abolished FEN-1 binding. Unexpectedly, we found that LC59A PCNA mutant bound UNG2 as efficiently as wild-type PCNA during size exclusion chromatography (Fig. 6). However, the ability of LC59A PCNA to stimulate UNG2 activity on calf thymus [*uracil*-³H]DNA decreased (58%) relative to wild-type PCNA. We observed that at relatively low concentrations, LC59A PCNA was more stimulatory to UNG2 activity than wild-type PCNA (Fig. 6B); however, the stimulatory effect of LC59A PCNA saturated at approximately 90 pmol, whereas that of wild-type PCNA saturated at approximately 800 pmol. These data indicate that the K_d of the UNG2-LC59A-PCNA complex was lower than that of the UNG2-PCNA complex, suggesting that the tighter interaction with LC59A PCNA may have interfered with the UNG2 search mechanism. It is also possible that UNG2 may have two PCNA-binding sites in analogous fashion to *S. cerevisiae* Apn2. Unk et al. [49] reported that in the absence of DNA, Apn2 bound to the IDCL domain of PCNA; however, when PCNA was loaded onto a DNA primer-template junction, the C-terminal domain of PCNA-mediated Apn2 binding. Similarly, PCNA may serve to recruit UNG2 through its N-terminal domain to sites of DNA synthesis. Following this initial binding event, UNG2 may change conformation or re-align to bring the core catalytic domain in closer contact with the DNA-bound PCNA and the newly synthesized DNA. Whether the UNG2-PCNA interaction is actually bi-modal awaits future experimentation.

We and others [39] observed that Mg^{2+} stimulated the uracil-DNA glycosylase activity of UNG2 but not UNGΔ84, indicating the N-terminal domain was necessary for stimulation. Kavli et al. [39] reported that UNG2 activity on double-stranded DNA was stimulated nearly 10-fold by 10 mM Mg^{2+} , whereas we observed a more modest two-fold effect. To investigate the effect of Mg^{2+} on UNG2 activity further, we created a linear 2840 bp [*uracil*-³H]DNA substrate using PCR and conducted a time course of uracil-DNA glycosylase activity in the absence and presence of 7.5 mM $MgCl_2$. When the initial rates (<10% substrate digestion) of uracil excision were compared, the presence of $MgCl_2$ stimulated the rate of uracil excision 27-fold relative to the control (data not shown). We speculate that the standard calf thymus [*uracil*-³H]DNA substrate first used to measure the effect of $MgCl_2$ contained various nicks and discontinuities caused by DNase I activation and nick-translation/strand displacement by Pol I that may have inhibited UNG2 processivity relative to the continuous, homogenous DNA substrate produced by PCR. Kavli et al. [39] found that 7.5 mM $MgCl_2$ reduced the K_m of UNG2 for a single-stranded uracil-containing DNA substrate from 13.8 to 0.1 μM . Clearly, the nature of the DNA substrate has a significant effect on the influence of Mg^{2+} on UNG2 activity.

Here we report that Mn^{2+} had a similar effect as Mg^{2+} on UNG2 activity on double-stranded DNA, and demonstrate that Fe^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} were inhibitory. The nature of this inhibition is unclear, since Co^{2+} and/or Zn^{2+} were reported to stimulate the activity of DNA repair enzymes such as *E. coli* Nfo [54] and Fpg [55], and $ZnSO_4$ has been included in the reaction buffer of human thymine-DNA glycosylase [56]. Addition of $MgCl_2$ to the assay buffer did not alter the circular dichroism spectrum of purified UNG2 (data not shown). It is possible that Fe^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , and Zn^{2+} disrupt the DNA binding activity of the core catalytic domain or inhibit uracil flipping and/or excision. Interestingly,

we found that addition of 20 mM EDTA to UNG2 activity assays resulted in a level of stimulation slightly greater than that induced by MgCl₂, and that the combination of 7.5 mM MgCl₂ and 20 mM EDTA was more stimulatory than MgCl₂ alone (data not shown). It is possible that EDTA removed inhibitory divalent metal cations from UNG2 that had bound during production in *E. coli* or purification (Ni-NTA agarose), scavenged metal cations bound to the DNA substrate, or that it formed a complex with the rather basic ($pI \approx 9.5$) UNG2. How metal cations affect the many roles of the UNG2 N-terminal domain awaits further elucidation.

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Fig. 1. Purity of proteins used in this study. (A) Human nuclear uracil-DNA glycosylase (UNG2) and (B) human wild-type PCNA (wt) and the L126D/I128E mutant of PCNA (LC59A) were purified as described under Section 2 and an portion (3 μ g) of each preparation was subjected to 12.5% SDS-polyacrylamide gel electrophoresis and visualized by staining with Coomassie Brilliant Blue G-250. The mobilities of the molecular weight standards (MWS) and tracking dye (TD) are indicated by arrows.

Fig. 2. Effect of metal ions on UNG2 activity. Standard uracil-DNA glycosylase reactions were conducted using UNG2 (solid bars) and UNG δ 84 (open bars) as described under Section 2. Enzyme activity was normalized to that observed in the control reactions (100%) conducted in the absence of metal ions. Error bars indicate the standard error of duplicate reactions.

Fig. 3.

Analysis of UNG2 binding to immobilized PCNA using Ni-NTA agarose affinity chromatography. (A) UNG2/PCNA standard curve. Mixtures (50 μ l) of PCNA (200 pmol) and various amounts of UNG2 (0, 50, 100, 150, 200, and 300 pmol) were prepared to give UNG2/PCNA molar ratios of 0, 0.25, 0.50, 0.75, 1.0, and 1.5. Samples (10 μ l) of the mixtures were analyzed by 12.5% SDS-PAGE followed by staining with Coomassie Brilliant Blue G250 (UNG2/PCNA sample stoichiometry indicated over each lane of the gel image, upper panel). After destaining, the intensity of the protein bands was quantified by densitometry using a ChemiGenius^{2XE} digital imaging system. The ratio of the intensities of the UNG2 and PCNA bands in each lane was determined and graphed relative to the stoichiometry of the UNG2/PCNA mixtures (lower panel). (B) Titration of PCNA with UNG2. His₆-tagged wild-type PCNA (200 pmol) was mixed with various amounts of UNG2 (0, 50, 100, 200, 400 and 600 pmol, lanes 1–6, respectively) and incubated at room temperature for 10 min. The mixtures (50 μ l) were applied to Ni-NTA agarose columns, which were washed and subsequently eluted with SDS sample buffer as described under Section 2. Aliquots (20 μ l) of the elution fractions were analyzed by 12.5% SDS-PAGE and

staining with Coomassie Brilliant Blue G-250 (upper panel). After destaining, the ratio of the UNG2/PCNA band intensities was quantified as described above, and the amount of UNG2 bound was determined from the standard curve shown in (A). The amount of UNG2 bound is shown as a function of the amount of UNG2 applied (lower panel). (C) Effect of NaCl on the UNG2–PCNA interaction. Mixtures of UNG2 (250 pmol) and PCNA (250 pmol) were incubated in buffer containing either 0, 25, 50, 75, 100 or 200 mM NaCl as indicated, and applied to an Ni-NTA agarose column equilibrated in the same buffer as the sample. After washing with the appropriate buffer, the columns were eluted with SDS-buffer and the eluate analyzed by SDS-PAGE, Coomassie staining, and digital imaging (gel image, upper panel). The ratio of the UNG2/PCNA band intensities was determined in the various elution fractions, and the amount of UNG2 bound expressed as a percentage of the amount of UNG2 detected at 0 mM NaCl (lower panel). (D) Effect of MgCl₂ concentration on the UNG2–PCNA interaction. Mixtures of UNG2 (250 pmol) and PCNA (250 pmol) were incubated in buffer containing either 0, 2.5, 5.0, 7.5, or 10 mM MgCl₂, and applied to an Ni-NTA agarose column equilibrated in the same buffer. After washing with the appropriate buffer, the columns were eluted with SDS-buffer and the eluate analyzed by SDS-PAGE, Coomassie staining, and digital imaging as described above. An image of the SDS-polyacrylamide gel is shown in the upper panel. The amount of UNG2 bound expressed as a percentage of the amount of UNG2 detected at 0 mM MgCl₂ is shown as a function of MgCl₂ concentration (lower panel).

Fig. 4.

Size exclusion chromatography of a UNG2-PCNA mixture. Samples (50 μ l) containing either 1 nmol PCNA (A), 1 nmol UNG2 (B), 1 nmol PCNA + 250 pmol UNG2 (C), 1 nmol PCNA + 250 pmol UNG2 + 100 mM NaCl (D), or 1 nmol PCNA + 250 pmol UNG Δ 84 (E) were incubated on ice for 10 min and applied to a Sephacryl-300 column as described under Section 2. Protein concentration was determined by the Bradford reaction (open circles, Absorbance 595 nm) using the Bio-Rad Protein Assay reagent. PCNA was detected by immunodot blot analysis (solid circles, panel A), and UNG2 and UNG Δ 84 proteins were detected by the standard uracil-DNA glycosylase activity assay (units/fraction of catalytic activity, solid circles, panels B, C, D and E) as described under Section 2. The location of the PCNA, UNG2, and UNG Δ 84 elution peaks are denoted by Roman numerals I, II, and III, respectively.

Fig. 5. Effect of PCNA on UNG2 activity. (A) The catalytic activity of UNG2 (solid circles) and UNG Δ 84 (open circles) in the presence of PCNA (0, 30, 90, 270, 810, 1620 pmol) was measured using the standard assay described under Section 2. (B) The effect of PCNA (0, 30, 90, 270, 810, 1620, 2430 pmol) on UNG2 activity in the absence (solid squares) and presence (open squares) of 7.5 mM MgCl₂.

Fig. 6. Interaction of UNG2 with the PCNA LC59A mutant. (A) Size exclusion chromatography of a UNG2–PCNA LC59A mixture. PCNA LC59A (1 nmol) was incubated with UNG2 (250 pmol) and subjected to size exclusion chromatography as described in the legend to Fig. 4. (B) The effect of wild-type PCNA (solid circles) and mutant LC59A PCNA (open circles) on the catalytic activity of UNG2 was measured as described in the legend to Fig. 5A.

Fig. 7.

Addition of replication factor C to UNG2–PCNA reactions. (A) Effect of linear vs. circular [*uracil*-³H]DNA substrate on UNG2 catalytic activity in the presence of PCNA. Form I M13 [*uracil*-³H] DNA was prepared in a primer extension reaction and isolated by cesium-chloride ethidium bromide density centrifugation as described under Section 2. Form III M13 [*uracil*-³H] DNA was prepared from Form II M13 [*uracil*-³H] DNA by *Eco*RI restriction endonuclease digestion. UNG2 was reacted with linear (black bars) or circular (white bars) M13 [*uracil*-³H] DNA substrate in the absence and presence of PCNA (810 pmol) as indicated, and the amount of ³H uracil released was quantified as described in the legend to Fig. 5. (B) Effect of RFC on UNG2 catalytic activity in the presence and absence of PCNA. UNG2–PCNA reaction mixtures containing either Form I or Form III M13 [*uracil*-³H] DNA, or nicked-translated calf thymus [*uracil*-³H]DNA as indicated, were incubated at 37 °C for 30 min in the absence (white bars) or presence (black bars) of RFC (1.9 µg) and the amount of ³H uracil excised was quantified as described in the legend to Fig. 5.