Post-replicative base excision repair in replication foci

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Base excision repair (BER) is initiated by a DNA glycosylase and is completed by alternative routes, one of which requires proliferating cell nuclear antigen (PCNA) and other proteins also involved in DNA replication. We report that the major nuclear uracil-DNA glycosylase (UNG2) increases in S phase, during which it co-localizes with incorporated BrdUrd in replication foci. Uracil is rapidly removed from replicatively incorporated dUMP residues in isolated nuclei. Neutralizing antibodies to UNG2 inhibit this removal, indicating that UNG2 is the major uracil-DNA glycosylase responsible. PCNA and replication protein A (RPA) co-localize with UNG2 in replication foci, and a direct molecular interaction of UNG2 with PCNA (one binding site) and RPA (two binding sites) was demonstrated using two-hybrid assays, a peptide SPOT assay and enzyme-linked immunosorbent assays. These results demonstrate rapid post-replicative removal of incorporated uracil by UNG2 and indicate the formation of a BER complex that contains UNG2, RPA and PCNA close to the replication fork.

Keywords: proliferating cell nuclear antigen/replication foci/replication protein A/uracil-DNA glycosylase

Introduction

Base excision repair (BER) of DNA is initiated by a DNA glycosylase unique for damage to a certain base or, more commonly, a group of related damaged bases (reviewed in Seeberg *et al.*, 1995; Krokan *et al.*, 1997; Glassner *et al.*, 1998). Uracil-DNA glycosylase (UDG or UNG) initiates BER for removal of uracil resulting from deamination of cytosine in DNA or misincorporation of dUMP. The *UNG* gene (Haug *et al.*, 1996) encodes mitochondrial (UNG1) and nuclear (UNG2) forms of uracil-DNA glycosylase using different promoters and alternative splicing (Nilsen *et al.*, 1997; Haug *et al.*, 1998). UNG1 and UNG2 have unique N-terminal regions required for subcellular sorting (Nilsen *et al.*, 1997; Otterlei *et al.*, 1998), while the

structurally and biochemically well-characterized catalytic domain (Mol *et al.*, 1995; Slupphaug *et al.*, 1996; Parikh *et al.*, 1998) is common for the two forms. Other uracil-DNA glycosylases have also been reported, including a thymine(uracil)-DNA glycosylase (TDG) with a strong preference for T or U mispaired with G (Nedderman and Jiricny, 1994), a cyclin-like uracil-DNA glycosylase (Muller and Caradonna, 1991) and a very recently reported uracil-DNA glycosylase (SMUG1) that like UNG proteins prefers uracil in single-stranded DNA as substrate (Haushalter *et al.*, 1999). It has not yet been determined whether the different uracil-DNA glycosylases have distinct or overlapping physiological functions.

The apurinic/aprimidinic (AP) site generated by monofunctional DNA glycosylases is cleaved by a 5'-AP-endonuclease (Rothwell and Hickson, 1997). Recently, it was shown that human 5'-AP-endonuclease (HAP1) displaces both UNG proteins (Bharati *et al.*, 1998; Parikh *et al.*, 1998) and TDG (Waters *et al.*, 1999) from the AP site to which both glycosylases bind tightly. This significantly enhances DNA glycosylase activities since dissociation from the AP site is a rate-limiting step. At least two pathways carry out the subsequent steps in nuclear BER. In one pathway, removal of deoxyribose 5-phosphate is carried out by a deoxyribosephosphodiesterase (dRpase) activity, probably contributed by DNA polymerase β (Srivastava *et al.*, 1998). Then the single nucleotide gap is filled in by DNA polymerase β aided by XRCC1, and the nick is ligated by DNA ligase III (Kubota *et al.*, 1996; Nicholl *et al.*, 1997). In an alternative BER pathway, the repair patch is larger and the process requires proliferating cell nuclear antigen (PCNA) (Matsumoto *et al.*, 1994; Frosina *et al.*, 1996), the structure-specific nuclease DNase IV (Fen1) and DNA ligase I (DeMott *et al.*, 1996; Klungland and Lindahl, 1997; Kim *et al.*, 1998). Furthermore, UNG protein has been shown to interact with the 34 kDa subunit of replication protein A (RPA2) (Nagelhus *et al.*, 1997) and, recently, RPA was reported to stimulate long patch BER *in vitro* (DeMott *et al.*, 1998). These results suggest a role for RPA in long patch BER. It is not clear whether the two pathways of BER have distinctly different functions.

In eukaryotes, DNA replication takes place in discrete replication foci (Nakamura *et al.*, 1986; Mills *et al.*, 1989) that vary in morphology during S phase and contain a number of proteins involved in replication, including DNA polymerase δ (Simbulan-Rosenthal *et al.*, 1996; reviewed in Leonhardt and Cardoso, 1995), but not DNA polymerase β (Yamamoto *et al.*, 1984; Li *et al.*, 1993; Applegren *et al.*, 1995). Here we present data strongly indicating that a BER pathway also operates in replication foci. Incorporated uracil is removed within a few minutes by UNG2 that co-localizes with RPA and PCNA in replication foci. Furthermore, UNG2 has two binding sites

Fig. 1. Localization of UNG2 and RPA2 in HaCaT cells during the cell cycle (after release from serum starvation). (A) UNG protein distribution at 0 h (G_0/G_1 phase) 20 h (late G_1 phase), 27 h (early/mid S phase), 30 h (mid S phase) and 32 h (late S phase/early G_2 phase), respectively, after serum addition. (B) Distribution of RPA2 in the same cells at corresponding times. (C) Incorporation of [3 H]thymidine (25 min pulse-labelling) into HaCaT cells at different times after release from serum starvation.

for RPA and one binding site for PCNA. This suggests that a long patch DNA polymerase δ /PCNA-requiring pathway for BER takes place in a BER complex containing UNG2, PCNA and RPA. This complex must be located close to the replication fork in replication foci and may be tailored for post-replicative removal of misincorporated dUMP residues.

Results

Expression of UNG2 in the cell cycle

To study the pattern of expression of UNG2 in the cell cycle, HaCaT cells were synchronized by serum starvation and examined by immunostaining for UNG protein at different time points after addition of complete medium. The antibodies used react with both nuclear UNG2 and mitochondrial UNG1, and the observed cytoplasmic staining most likely represents mitochondrial UNG1. Only minor amounts of nuclear UNG2 were observed in nuclei of G_0/G_1 cells, but increased very markedly in S phase. In contrast, the nuclear content of RPA in the same preparations was essentially cell cycle independent (Figure 1). In S phase cells, the immunostaining demonstrated a spotted distribution of UNG2, and this distribution was even more pronounced for RPA in S phase (27–30 h). Others have shown that RPA is located in similar spots which represent replication foci (Brenot-Bosc *et al.*, 1995; Murti *et al.*, 1996). Compartments resembling nucleoli were stained weakly for both proteins. As a complement to these immunostaining studies, we carried out transfection experiments with synchronized HaCaT cells. We found that the low content of nuclear UNG2 in the G_1 phase

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Fig. 2. Effects of neutralizing anti-UNG antibodies on *in vitro* DNA replication in isolated HeLa cell nuclei and on UNG activity, and unstable incorporation of [3 H]dUMP into DNA. (A) Incorporation of [3 H]dTTP into isolated nuclei in the absence (●) or presence (○) of neutralizing antibodies to UNG proteins. (B) UNG activity in isolated nuclei in the absence (●) or presence (○) of neutralizing antibodies to UNG proteins and UNG activity in sonicates of isolated nuclei in the absence (◆) or presence (◇) of neutralizing antibodies. UNG activity was measured using added [3 H]uracil-containing DNA. (C) Incorporation of [3 H]dUTP into isolated nuclei in the absence (●) or presence (○) of neutralizing antibodies to UNG proteins.

was not due to a lack of nuclear translocation capacity in this cell cycle phase because when cells were transfected early in the G_1 phase, UNG2 expressed in UNG2–green fluorescent protein (EGFP) fusion products rapidly translocated to nuclei also in the G_1 phase, with no visible accumulation in the cytoplasm (data not shown). Thus, a cell cycle-dependent variation in biosynthesis of UNG2 is the most likely explanation for the variation in UNG2 content during the cell cycle, in agreement with recent studies on UNG1 and UNG2 mRNA during the cell cycle (Haug *et al.*, 1998).

UNG2 removes uracil from misincorporated dUMP residues in an immediate post-replicative process

To examine the possible role of UNG2 in removal of uracil from misincorporated dUMP residues, we used an *in vitro* system based on isolated nuclei from HeLa cells (Figure 2). This replication system faithfully and efficiently elongates already initiated DNA molecules and initiates new Okazaki fragments, but is probably unable to initiate DNA replication at replication origins (Krokan *et al.*, 1975a,b). Incorporation of radioactive dNTPs by isolated

nuclei correlates directly to the DNA synthetic activity of the cells from which the nuclei are isolated, and incorporation is reduced by 90–95% in nuclei outside of the S phase (Krokan and Eriksen, 1977). Neutralizing antibodies that essentially abolished UNG activity in nuclei or nuclear sonicates (Figure 2B) had no effect on DNA synthesis at the replication fork, as measured by [3 H]TTP incorporation (Figure 2A). This demonstrates that UNG2 is not required for DNA chain elongation or initiation of Okazaki fragments. When the DNA replication mixture contained [3 H]dUTP in addition to the dNTPs, [3 H]uracil incorporated was unstable and started to decrease after an initial rapid incorporation phase (Figure 2C). The initial elongation rate of the *in vitro* system is ~30% of the *in vivo* rate (Krokan *et al.*, 1975a). The equilibrium between replicative incorporation of dUMP and excision of uracil from dUMP (Figure 2C) may be reached already after ~1–2 min (5 min *in vitro*) of incorporation, after which the rate of excision exceeds the rate of incorporation. Incorporated uracil was protected from excision by UNG-neutralizing antibodies (Figure 2C), demonstrating that UNG2 is responsible for removal of uracil and that it has access to nascent DNA immediately after new deoxyribonucleotides are incorporated. This indicates the presence of UNG2 in replication foci and that a post-replicative BER pathway takes place in replication foci.

Co-localization of UNG2, RPA and PCNA in replication foci

Nuclear localization of UNG2, RPA and PCNA was examined by confocal microscopy (Figure 3). To identify whether nuclear spots observed after immunostaining of RPA or UNG2 in S phase cells were replication foci, logarithmically growing HeLa cells were transfected with plasmid pUNG2EGFP (green nuclei) prior to incorporation of bromodeoxyuridine (BrdUrd). Staining of replication foci by antibodies against BrdUrd (red nuclei) shows that BrdUrd co-localizes with UNG2 spots, demonstrating that UNG2 is localized in replication foci. Both immunostaining and transfection with a construct expressing the UNG2EGFP fusion protein indicate that UNG2 is not localized exclusively to replication foci. Furthermore, staining of pUNG2EGFP-transfected cells with antibodies against either RPA or PCNA shows that UNG2 also co-localizes with RPA and PCNA within replication foci. The overlapping of UNG2 with either BrdUrd incorporation, RPA or PCNA is visualized as yellow spots (right panels). UNG2 was not localized to spots resembling replication foci in cells that did not incorporate BrdUrd; thus this morphology is S-phase specific (data not shown). In conclusion, these experiments demonstrate that UNG2, PCNA and RPA co-localize in replication foci, although some UNG2 is also found outside of replication foci.

Molecular interactions between UNG2 and RPA

We have shown previously that a region within the N-terminal UNG1 residues 29–75 (most of which are common to UNG1 and UNG2) binds RPA2, a 34 kDa subunit of trimeric RPA (Nagelhus *et al.*, 1997). Two-hybrid analysis specifies the region essential for interaction with RPA2 more accurately to residues 67–85 (Figure 4A). The residues in RPA2 required for UNG2 binding were also mapped by two-hybrid analysis and were found to

be in the C-terminal part beyond position 163 (Figure 4B). In the peptide SPOT assay, binding of trimeric RPA to peptide UNG2/13 narrowed down the core binding region further to residues 73–84 (Figure 5A). The SPOT assay also suggests that RPA has a second binding site in UNG2, as indicated by binding to peptide UNG2/2, corresponding to UNG2 residues 7–18 (L₇Y₈S₉F₁₀F₁₁S₁₂P₁₃S₁₄P₁₅A₁₆R₁₇K₁₈, site 1). Enzyme-linked immunosorbent assays (ELISAs) designed to quantify interaction of UNG2 with RPA revealed that full-length UNG2 bound several-fold more trimeric RPA than a truncated form lacking the 44 N-terminal amino acids, while a truncated form lacking the 94 N-terminal amino acids did not bind RPA (Figure 5C). This supports the presence of a second RPA interaction site N-terminal in UNG2. We have not been able to demonstrate binding to this UNG2 site in two-hybrid analysis using each of the three RPA subunits independently. Possibly the binding to the second site requires trimeric RPA, or a conformation not easily adapted by the expressed isolated subunits.

Molecular interactions between UNG2 and PCNA

A putative PCNA-binding site was identified in UNG2 using a profile searching technique of protein sequence databases (Warbrick *et al.*, 1998). The potential of PCNA to interact with UNG2 was supported further by the observed co-localization of these proteins. Therefore, to characterize this interaction further, a set of UNG2-expressing plasmids (UNG2, UNG2_{1–48} and UNG2_{45–151}) was tested against plasmids expressing PCNA from various species (human, *Drosophila* and *Schizosaccharomyces pombe*) in the two-hybrid system. The UNG2 constructs were also tested against plasmid expressing full-length Fen1, a protein known to bind PCNA. Two forms of test were carried out: either pairs of plasmids were co-transformed into *Saccharomyces cerevisiae* strain Y190 or pairs of plasmids were tested in diploid strains resulting from crossing transformed strains of Y190 and Y187 (see Materials and methods for details). These slightly different tests gave essentially identical results. The results in Table I show that both *S.pombe* and *Drosophila* PCNA are capable of interacting specifically with full-length UNG2. Both forms of PCNA interact with the N-terminal 48 amino acids, whilst deletion of this region abolishes the interaction, thus indicating that amino acids 1–44 represent a region of the protein both necessary and sufficient for the interaction. This region corresponds to the UNG2-specific sequences within the protein (Nilsen *et al.*, 1997). None of the UNG2-expressing plasmids interacted with Fen1, p53 or lamin, indicating that the interaction is specific. Surprisingly, however, UNG2 did not interact with human PCNA in this system. The reason for this result is not clear: the positive control test for interaction with *S.pombe* PCNA (*spPCNA*) shows that *S.pombe*, *Drosophila* and human PCNA were all expressed and were all capable of interacting with *spPCNA* in this series of two-hybrid tests. However, results from the ELISAs (Figure 6) which quantify binding of different N-terminally deleted recombinant UNG2 proteins to human recombinant PCNA demonstrate that UNG2 does bind human PCNA. Furthermore, the ELISA data indicate that the 10 N-terminal amino acids in UNG2 are essential for binding to PCNA.

Fig. 3. Co-localization of UNG2-EGFP fusion protein and BrdUrd, RPA and PCNA. UNG2-EGFP fusions are shown in the left side panels below the green arrow. Visualization of BrdUrd, RPA and PCNA with specific antibodies and rhodamine (red) is shown in the three middle vertical panels. The three panels at the right below the yellow arrow demonstrate co-localization of UNG2-EGFP with BrdUrd (top), RPA (middle) or PCNA (bottom) after superimposition of rhodamine staining and green fluorescence. In each case, the resulting yellow spots represent a direct visualization of co-localization.

Although we did not observe interaction in the two-hybrid system between full-length human PCNA and UNG2, a very weak positive interaction was observed in the two-hybrid system with full-length UNG2 and amino acids 1–255 of human PCNA. This was not seen with other C- and N-terminal deletion clones of PCNA tested (data not shown). PCNA_{1–255} lacks the acidic C-terminus of PCNA, a region that has been implicated in the binding of a peptide containing the PCNA-binding domain from p21^{Cip1/WAF1} (Gulbis *et al.*, 1996). However, the full-length p21 protein binds to human PCNA in the two-hybrid system, and loss of the C-terminus of human PCNA does not significantly affect the interaction (Warbrick *et al.*, 1995). One possible explanation for the observed results with UNG2 is that removal of the C-terminus of PCNA subtly affects the conformation of the complex binding site on PCNA, thus allowing UNG2 to bind with slightly higher affinity. The lack of interaction between UNG2 and full-length human recombinant PCNA in the two-hybrid system may also be caused by intrinsic problems

related to the two-hybrid system itself, such as masking of sites of interaction by the fusion partner, and possibly competition for binding by several other PCNA-binding proteins.

Studies on UNG2-PCNA interactions using short peptides from the UNG2 N-terminal region

As the N-terminus of human UNG2 is capable of interacting with PCNA in the two-hybrid system, and the deletion of the 10 N-terminal amino acids in UNG2 severely reduced the binding of UNG2 to PCNA in the ELISA, we next examined whether small N-terminal regions of UNG2 were capable of binding to PCNA *in vitro*. Synthetic peptides of 20 amino acids corresponding to the PCNA-binding consensus regions in human, mouse and *S.cerevisiae* uracil-DNA glycosylases were linked to biotin through an SGSG linker at their N-terminus (Nilsen *et al.*, 1997; Percival *et al.*, 1989). A p21^{Cip1/WAF1}-derived peptide whose interaction with PCNA has been characterized previously was also included in the tests

Fig. 4. Two-hybrid analysis of the interaction of UNG2 with human RPA2. (A) Interaction of different deletion clones of UNG2 with RPA2. (B) Interaction of different deletion clones of RPA2 with UNG2.

(Warbrick *et al.*, 1995; Gulbis *et al.*, 1996). In each case, a peptide with the conserved glutamine residue substituted with alanine (Q4A) was tested. In order to determine whether the peptides were capable of binding to PCNA, they were conjugated to streptavidin-agarose beads and incubated with either HeLa or *S.pombe* cell extracts. Figure 7A and B shows that all the peptides with 'wild-type' sequences were capable of binding strongly to PCNA in both human and *S.pombe* extracts. Although the Q4A substitution did not substantially affect binding to the p21 peptide in human extracts, binding was significantly reduced in *S.pombe* extracts. The substitution in the human UNG2-derived peptide did not affect binding in human or *S.pombe* extracts, while in the mouse UNG2-derived peptide, only human PCNA binding was affected. In contrast, substitution in the yeast UNG2-derived peptide significantly affected binding in both human and *S.pombe* extracts. These results suggest that species-specific differences in binding exist, even in these small, 20 amino acid peptide sequences. Competition assays were performed to examine whether these peptides are binding to the same region of PCNA as human p21. These results shown in Figure 7C indicate that although the unbiotinylated peptide KRRQTSMTDFYHSKRRLIFS (p21-derived peptide) competes only very weakly for the same sequence bound to beads, it was able to compete effectively for the binding of PCNA to the UNG2-derived peptides tested, compared with a control peptide. This evidence, together with the primary sequence similarity between these peptides, strongly suggests that the UNG2-derived peptides are binding to the same site within PCNA as p21.

Discussion

We present evidence that one of the identified pathways for BER takes place in replication foci in which UNG2 co-localizes with RPA and PCNA. Furthermore, UNG2

Fig. 5. Binding of trimeric RPA to UNG2 or UNG2-derived peptides. (A) SPOT assay for binding of synthetic, cellulose membrane-attached UNG2 peptides to trimeric RPA. Peptide sequences (1–14) are given below and together cover the 90 N-terminal residues (1 representing the first 12 residues in UNG2, and 14 representing residues 78–90). Note the six amino acid overlap for consecutive peptides. The antibody used for detection of RPA was a RPA2-specific antibody. In (B), the same membrane was stripped and incubated with RPA2-specific antibodies in the absence of RPA. (C) ELISA showing binding of RPA to coats of UNG2₉₅₋₃₁₃, UNG2₄₅₋₃₁₃ and UNG2₁₋₃₁₃ (complete UNG2).

Fig. 6. Binding of full-length and N-terminally deleted UNG2 proteins to ELISA plates coated with human recombinant PCNA. Full-length UNG2 (black), UNG2₁₁₋₃₁₃ (grey) and UNG2₄₅₋₃₁₃ (white).

Table 1. Results of two-hybrid interaction testing between UDG and PCNA expression constructs

pACT- pAS2-	UNG2	UNG2 ₁₋₄₈	UNG ₄₅₋₁₅₁	spPCNA	SNF4
spPCNA	++	+	–	++	–
<i>Drosophila</i> PCNA	+	(+)	–	+	–
Human PCNA	–	–	–	++	–
Fen1	–	–	–	++	–
p53	–	–	–	–	–
Lamin	–	–	–	–	–

Pairs of plasmids were tested against each other for activation of reporter constructs as described in the text. Only co-transformants which expressed both reporter constructs His3 and LacZ were judged to represent positive interaction. The results in this table represent the expression of LacZ as judged by the blue colour of cells following a filter lift assay in the presence of the LacZ substrate X-gal: ++ indicates positive in 30 min or less; + indicates positive after 2 h; (+) indicates a weak blue colour after 2 h; and – indicates that no reaction was observed.

engages in molecular interactions with these proteins through its N-terminal sequence, suggesting the formation of a BER complex involving UNG2, PCNA and RPA in replication foci. The regions involved in molecular interactions, as well as conserved sequence motifs in UNG2, are summarized in Figure 8. We also demonstrate that removal of incorporated dUMP is carried out mainly by UNG2, since neutralizing antibodies to UNG2 efficiently protected uracil from excision. This would seem to rule out a major function for TDG, which actually removes uracil more efficiently than thymine (Neddermann and Jiricny, 1994), the reported cyclin-related uracil-DNA glycosylase (Muller and Caradonna, 1991) and SMUG1 (Haushalter *et al.*, 1999) in the removal of incorporated uracil residues. The rapid removal of incorporated uracil is consistent with the previous observation that incorporation of dUMP into growing DNA strands in isolated nuclei from S phase cells results in fragmentation of DNA to sizes smaller than normal Okazaki fragments within 1 min after the incorporation of dUMP (Wist *et al.*, 1978). It is also consistent with the preferential association of uracil-DNA glycosylase activity with replicating SV40 chromatin (Krokan, 1981).

It is possible that removal of replicatively incorporated uracil (in U:A pairs) may take place by a mechanism different from that which removes uracil from U:G mispairs resulting from cytosine deamination. The former repair process must be post-replicative, although not necessarily immediate, while the latter must be pre-replicative, although not necessarily immediate, in order to prevent GC to AT transition mutations. Interestingly, DNA polymerase β is not present in multiprotein replication complexes/replication foci (Yamamoto *et al.*, 1984; Li *et al.*, 1993; Applegren *et al.*, 1995). This indicates that the single nucleotide pathway requiring DNA polymerase β is not central in the immediate post-replicative removal of incorporated uracil residues in replication foci. Our demonstration of direct molecular interactions of UNG2 with PCNA and RPA and their presence in replication foci instead suggest that the 2–8 nucleotide patch, PCNA-requiring BER pathway may operate in replication foci. This is also supported by the demonstrated presence of other factors implicated in this BER pathway (DNA

Fig. 7. Small regions of uracil-DNA glycosylases from different species (UDG) are capable of binding to PCNA from extracts of human cells or *S.pombe* *in vitro*. (A) The results using HeLa cell extract. (B) The results of a parallel experiment using *S.pombe* extract. The peptides used are described in Materials and methods, and represent N-terminal amino acids from human p21^{Cip1/WAF1} (p21), human UNG2 (UDGH), mouse UNG2 (UDGM) and *S.cerevisiae* UNG2 (UDGY). In each case, peptides were also used with conserved glutamine substituted with alanine (Q4A) (p21-A, UDGH-A, UDGM-A, UDGY-A). A peptide of unrelated sequence was used as a control for non-specific binding. (C) Competition assay. The ability of immobilized peptides to bind to PCNA was tested in the presence of either a p21-derived peptide (+), an unrelated control peptide (c) or the solvent DMSO (–).

polymerase δ , PCNA, DNA ligase I and RPA) in replication foci or multiprotein replication complexes (Yamamoto *et al.*, 1984; Bravo and Macdonald-Bravo, 1987; Li *et al.*, 1993; Simbulan-Rosenthal *et al.*, 1996). Possibly the single nucleotide pathway that utilizes DNA polymerase β , but not PCNA (Singhal *et al.*, 1995; Kubota *et al.*, 1996; Nicholl *et al.*, 1997), may operate through the entire cell cycle and may have as one function to remove uracil resulting from deamination of cytosine. Since DNA polymerases δ and ϵ may also fill in single nucleotide gaps in BER, albeit at strongly reduced rates (Stucki *et al.*, 1998),

Fig. 8. Overview of functional domains in UNG2 and inter-species conservation of protein interaction motifs. **(A)** A functional representation of UNG2. The 44 N-terminal residues unique to UNG2 are essential (but not sufficient) for complete nuclear targeting and have overlapping motifs involved in binding of RPA and PCNA. A second RPA-binding motif is found in the sequence common to UNG2 and UNG1, and is located immediately N-terminal to the start of the catalytic domain (residues 85–313). **(B)** Amino acid sequences of PCNA- and RPA-binding motifs in uracil-DNA glycosylases from different species, and in human p21. Conserved motifs required for PCNA binding are shown in bold.

pre-replicative BER in replication foci could utilize these DNA polymerases. Interestingly, the long-patch BER pathway may utilize either DNA polymerase β or δ (Klungland and Lindahl, 1997), or DNA polymerase ϵ (DeMott *et al.*, 1998). Thus, the long patch repair pathway may be more versatile than the single nucleotide pathway in that it may repair simple AP sites as well as modified AP sites, and may utilize different polymerases.

RPA has been shown to be involved in the start of replication, initial steps of nucleotide excision repair (NER) and in recombination repair (reviewed in Wold, 1997), and may be involved in BER (Nagelhus *et al.*, 1997; DeMott *et al.*, 1998). We have identified two regions involved in RPA binding in the N-terminal sequence of UNG2 outside the catalytic domain, and the presence of both sites strongly enhances binding of RPA to UNG2. Interestingly, both sites carry homology to short sequences in other DNA repair enzymes. The RPA-binding site in UNG2 from amino acids 73 to 84 has some homology to an RPA-binding region in XPA. The RPA-binding site close to the N-terminus overlaps with the PCNA-binding region in UNG2, which lies within the region of the protein specific to the nuclear form of the protein. The conserved PCNA-binding motif (QxxLxxFF) in this region conforms with the consensus (QxxL/I/MxxF/HF/Y) for PCNA binding found in a number of proteins involved in DNA repair, DNA replication, cell cycle control or DNA modification; these include p21, Fen1, XPG, DNA ligase I, replication factor C (RFC), DNA-(cytosine-5)-methyltransferase (MCMT) and G/T-mismatch-binding protein, GTBP/hMSH6 (Warbrick *et al.*, 1995, 1997, 1998; Nicolaides *et al.*, 1996; Chuang *et al.*, 1997; Gary *et al.*, 1997; Montecucco *et al.*, 1998). UNG2 contains the PCNA-binding motif at the extreme N-terminus, as is the case in DNA ligase I and the large subunit of RFC. Montecucco *et al.* (1998) have shown that the N-termini

of these proteins interact with PCNA, and can function to target heterologous proteins to replication foci within the nucleus. The PCNA-binding site is conserved in mouse UNG2 and also in UNG homologues from budding and fission yeast. In yeast, however, only a single form of the homologous protein exists, and sequence data suggest that these are functionally equivalent to the nuclear form (UNG2) in mammals. It recently has emerged that PCNA is capable of interacting with many proteins, a large proportion of which contain the PCNA consensus motif. Although PCNA is trimeric, a single trimer is not capable of binding to all these proteins simultaneously. It seems more likely that PCNA is engaged in the formation of dynamic complexes with a number of alternative proteins, forming a moving platform through which they can interact with DNA (Kelman and Hurwitz, 1998). We have also identified two RPA-binding sites within UNG2. Interestingly, the N-terminal RPA-binding site in UNG2 appears to overlap with the PCNA-binding motif. Such a close association of a PCNA-binding motif with another functional domain is also seen in p21 where a cyclin-dependent kinase inhibitory domain lies adjacent to the PCNA-binding region (Ball *et al.*, 1996). The relationship between the N-terminal RPA- and PCNA-binding domains in UNG2 is not clear and presently is under investigation.

Interaction of PCNA with DNA polymerase δ involves the exposed interdomain connector loop (amino acids 118–135) on the C-terminal side of the PCNA surface (Jonsson *et al.*, 1998; Zhang *et al.*, 1998). The residues in DNA polymerase δ involved in the binding have not been identified distinctly, and this polymerase does not carry the identified consensus motif for binding to PCNA. The binding site in PCNA for DNA polymerase ϵ has not been identified clearly, but is apparently different from the DNA polymerase δ -binding region (reviewed in Jonsson and Hübscher, 1997).

Materials and methods

DNA replication in isolated HeLa cell nuclei and access of antibodies to nuclear UNG2

In vitro DNA replication in nuclei (5×10^6 nuclei in 100 μ l assay mixture) isolated from exponentially growing HeLa S3 cells in monolayers was measured as described (Krokan *et al.*, 1975a,b) except that the incubation buffer contained 65 mM KCl instead of 65 mM NH_4Cl . The final concentration of radiolabelled dNTP ($[^3\text{H}]\text{dTTP}$, sp. act. 2.6 $\mu\text{Ci/nmol}$, or $[^3\text{H}]\text{dUTP}$, 2.4 $\mu\text{Ci/nmol}$) was 50 μM . When neutralizing polyclonal anti-UNG antibodies PU101 (Slupphaug *et al.*, 1995) were present (0.8 μg per reaction), nuclei were pre-incubated on ice with antibodies, or mock incubated, for 15 min prior to incubation at 37°C with assay mixture. Incorporated radioactivity was measured by scintillation spectrometry. To evaluate further nuclear access of the anti-UNG antibodies, nuclei or nuclear sonicates were incubated with DNA replication buffer lacking dNTPs, but instead supplemented with $[^3\text{H}]\text{uracil}$ -containing DNA. Released radiolabelled uracil was measured as described previously (Krokan and Wittwer, 1981).

Cell cycle studies

HaCaT cells (2×10^4 cells/well) were grown overnight on glass coverslips (14 mm) at 8% CO_2 and 37°C in Dulbecco's modified Eagle's medium with 1000 mg/l glucose, 10% fetal calf serum (FCS), 0.3 mg/ml glutamine, 0.1 mg/ml gentamicin and 2 $\mu\text{g}/\text{ml}$ fungizone. All reagents were from Gibco-BRL (Gaithersburg, MD). After 72 h of serum starvation, medium with FCS was added, and duplicate samples of cells pulse-labelled with $[^3\text{H}]\text{thymidine}$ (5 $\mu\text{Ci}/\text{ml}$ medium) for 25 min. Immunostaining was carried out at 0, 3, 8, 15, 18, 20, 23, 27, 30, 32 and 36 h after serum addition. $[^3\text{H}]\text{thymidine}$ incorporation was measured by scintillation spectrometry of trypsin-released cells harvested by a Titertec multiple cell harvester (Skatron A/S, Lier, Norway).

Protein fusion constructions, immunostaining, transient transfection and co-localization analysis by confocal microscopy

pUNG2EGFP was made as described previously (Nilsen *et al.*, 1997). Synchronized HaCaT cells and freely cycling HeLa cells were immunostained as described previously (Nagelhus *et al.*, 1995) using rabbit UNG-specific antibodies PU101, a mouse anti-RPA2 antibody (p34, 71-9A) and mouse anti-PCNA antibody PC10 (from ascites). BrdUrd (50 μM) incorporation (45 min in 37°C), fixation and detection with the mouse anti-BrdUrd antibody were done according to the recommendations of the manufacturer of the 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I (Boehringer Mannheim, Germany). Secondary antibodies used were biotinylated goat anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated streptavidin (Dako) (UNG2) or rhodamine (tetramethyl)-conjugated goat anti-mouse antibodies (Molecular Probes, Eugene, OR) for staining of UNG2, BrdUrd, RPA2 and PCNA. HeLa cells were transfected using calcium phosphate (Profection, Promega) according to the manufacturer's recommendations.

Transfected cells were examined using either a Bio-Rad MRC-600 confocal microscope (Figure 1) or a Leica TCS-NT digital scanning confocal microscope (Figure 3). For the Bio-Rad instrument, 488 nm (BHS) and 514 nm (GHS) excitation laser lines and a 60 $\times$ Nikon water immersion objective with NA = 1.2 were used. The 488 nm laser line was used for excitation of FITC/EGFP, and fluorescence was detected at $\lambda > 515$ nm (BHS-filter). Two-parameter confocal microscopy analysis was performed in consecutive scans with the 488 and 514 nm laser lines, respectively. FITC/EGFP and rhodamine fluorescence were detected at $525 \text{ nm} < \lambda_{\text{FITC/EGFP}} < 555 \text{ nm}$ (A2, BHS, PMT2) and $\lambda_{\text{rhodamine}} > 600 \text{ nm}$ (A2, GHS, PMT1), respectively. Pinhole sizes of 2/15 ($\lambda_{\text{FITC/EGFP}}$) and 12/15 ($\lambda_{\text{rhodamine}}$) were used for PMT2 and PMT1, respectively, in order to optimize the imaging for the specified fluorophores. For the Leica instrument, equipped with a 100 $\times$ /NA = 1.4 oil immersion objective, we used the 488 nm laser line for excitation of EGFP (detected at $530 \text{ nm} < \lambda_{\text{EGFP}} < 560 \text{ nm}$) and the 568 nm laser lines for the rhodamine fluorescence (detected at $> 590 \text{ nm}$). The pinhole diameter was kept at 1 μm . Images were exported to Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Yeast two-hybrid methods

Yeast reporter strain SFY526, used in the two-hybrid system, was co-transformed with plasmid vectors pGBT9 and pGADGH (MATCHMAKER Two-hybrid system, Clontech Laboratories Inc., Palo Alto, CA), in which constructs of UNG2 and p34 inserts were cloned,

and tested for β -galactosidase activity according to the manufacturer's manual. pGBT9UNG2₄₉₋₃₁₃, pGBT9UNG2₆₃₋₃₁₃, pGBT9UNG2₆₇₋₃₁₃, pGBT9UNG2₈₅₋₃₁₃ and pGBT9UNG2₉₄₋₃₁₃, which lack N-terminal amino acids as indicated, were made as described (Slupphaug *et al.*, 1995; Nagelhus *et al.*, 1997). pGBT9UNG2₁₋₄₈ was prepared by ligation of the 2436 bp *AatII*-*BglII* fragment of pGBT9UNG2 and the 3304 bp *AatII*-*SmaI* fragment of pGBT9. pGADGH-p34₅₋₂₇₀ was kindly provided by Dr K. Tanaka at Osaka University, Japan. pGADGH-p34₈₄₋₂₇₀ was prepared as previously described (Nagelhus *et al.*, 1997), and pGADGH-p34₁₃₆₋₂₇₀ was made by digestion at the *BclI* site in p34. The constructs pGADGH-p34₁₆₃₋₂₇₀, pGAD424-p34₁₉₅₋₂₇₀ and pGAD424-p34₂₃₃₋₂₇₀, as well as the construct pGBT9UNG1₂₉₋₈₃ (named pGBT9UNG2₄₅₋₉₂ in the present study), were made by introducing restriction sites by site-directed mutagenesis. The interaction between SNF1-Gal4-BD and SNF4-Gal4-AD in the two-hybrid system was used as positive control (Chien *et al.*, 1991), and plasmid vectors without insert were used as negative control. The *EcoRI*-*Sall* and the *EcoRI*-*PstI* fragments from pGBT9UNG2 and pGBT9UNG2₁₋₄₈, respectively, were subcloned into the pAS2-1 vector. The *SfiI*-*Sall* fragment from pAS2UNG2 and the *SfiI*-*PstI* fragment from pAS2UNG2₁₋₄₈ were ligated to the *SfiI*-*BamHI*-blunted pACT-2 vector in order to make pACTUNG2 and pACTUNG2₁₋₄₈, respectively. The *NcoI* fragment from UNG1₂₉₋₃₀₄ previously described (Slupphaug *et al.*, 1995) was ligated into the *NcoI* site of pACT-2 in order to prepare pACTUNG2₄₅₋₃₁₃. The plasmids expressing human, *Drosophila* and *S.pombe* PCNA have been described (Hall *et al.*, 1995; Warbrick *et al.*, 1995). The plasmid pAS-Fen1 was prepared by subcloning a full-length cDNA clone of human Fen1 from a pACT-Fen1 plasmid identified in a two-hybrid screening experiment. The plasmids pACT-SNF4, pAS-p53 and pAS-lamin have been described previously (Fields and Song 1989; Harper *et al.*, 1993). Growth and maintenance of *S.cerevisiae* were according to standard methods (Rose *et al.*, 1990). Transformation was carried out by the method of Gietz *et al.* (1992). The *S.cerevisiae* strain Y190 (*MAT α gal4 Δ gal80 Δ ade2-101 his3- Δ 200 leu2-3,112 trp1-901 ura3-52 cyh^R URA3::GAL1-lacZ LYS2::GAL1-HIS3*) was used, which expresses the reporter genes *lacZ* (*Escherichia coli*) and *HIS3* (*S.cerevisiae*) under the control of the *GAL1* promoter. Pairs of pAS- and pACT-derived plasmids were co-transformed into this strain, and the resultant transformants tested for reporter gene expression as described below.

For interaction mating experiments, pACT-derived plasmids were transformed into Y190, while pAS2-derived plasmids, expressing proteins as fusion with the DNA-binding domain of Gal4, were transformed into Y187 (*MAT α gal4 Δ gal80 Δ ade2-101 his3- Δ 200 leu2-3,112 trp1-901 ura3-52 URA3::GAL1-lacZ*). Pools of transformed cells in each case were grown on suitably selective medium, and then cross-stamped onto minimal medium with adenine, followed by incubation at 30°C to select for diploid cells containing both plasmids. Diploids thus derived were streaked onto selective minimal medium containing 50 mM 3-aminotriazole to test for His3 reporter expression. These cells were then tested for LacZ expression using a simple filter lift assay as previously described (Warbrick *et al.*, 1995). The plasmids with cDNA inserts used in the two-hybrid assay were all sequenced with TaqPRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit on an Applied Biosystems Model 373A DNA sequencing system to verify their structures.

Heterologous expression and purification of proteins

Recombinant expression and purification of UNG2₉₅₋₃₁₃ have been described previously (Slupphaug *et al.*, 1995). Purification of UNG2 (313 residues) expressed from full-length cDNA resulted in a preparation containing a mixture of full-length UNG2 protein (80%) and UNG2 lacking the N-terminal methionine residue (UNG2₂₋₃₁₃, 20%), as demonstrated by peptide sequencing. These could not be separated in our purification scheme. In addition, the purification yielded recombinant UNG2₈₋₃₁₃/UNG2₁₁₋₃₁₃ as a mixture. The purification and biochemical characterization of the full-length enzyme will be published separately. UNG2₄₅₋₃₁₃ was expressed and purified as described by Bharati *et al.* (1998). Human recombinant PCNA was a generous gift from Daniella Zhelev and Nikolai Zhelev at Cyclacel Ltd, Dundee, UK.

Enzyme-linked immunosorbent assays (ELISAs)

ELISA experiments for measuring RPA binding to different deletion clones of UNG2, and binding of different deletion clones of UNG2 to PCNA were carried out essentially as described (Nagelhus *et al.*, 1997). Different UNG proteins were coated [1 μg in 100 μl of phosphate-buffered saline (PBS) at 4°C overnight] onto microtitre plates. The wells were blocked with 5 mg/ml bovine serum albumin (BSA) in PBS before

RPA was added. Binding of RPA was quantified by the anti-mouse RPA2 antibody (p34, 71-9A), (Erdile *et al.*, 1990) and a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark). Similarly, 1 µg of PCNA in 100 µl of PBS was coated onto microtitre plates and wells blocked with 2 mg/ml gelatine solution in PBS (filtered through 0.2 µm). Different concentrations of the UNG2 preparation (containing 80% full-length UNG2 and 20% UNG2₂₋₃₁₃), UNG2₁₁₋₃₁₃ (containing 80% UNG2₁₁₋₃₁₃ and 20% UNG2₈₋₃₁₃) and UNG2₄₅₋₃₁₃ dissolved in PBS containing 1 mg/ml BSA were then added to wells coated with PCNA and blocked with gelatine, and to wells without PCNA but blocked with gelatine. Binding of UNG2 proteins was detected using the rabbit UNG-specific antibodies and an HRP-conjugated rabbit anti-mouse IgG (Dako, Denmark). The OD values represent the difference between binding of UNG proteins to PCNA coat and binding to the gelatine block only.

Binding of RPA to membrane-bound synthetic peptides

A panel of 14 synthetic, overlapping peptides corresponding to the N-terminal 88 amino acids of UNG2 was synthesized on a derivatized cellulose membrane (SPOTs, Genosys, Cambridge, UK). The membrane was blocked overnight at 4°C with 3% BSA in PBS, 0.1% Tween-20 (PBST). The subsequent steps were carried out at room temperature. The membrane was washed in PBST (10 min) and incubated for 60 min in 0.02 µg/ml RPA in PBS. After washing (3× 15 min in PBST), the membrane was incubated with anti-mouse RPA2 antibodies (p34, 71-9A) (1.1 µg/ml in PBST) for 30 min. Membranes were washed (3× 15 min) and then incubated for 30 min with peroxidase-conjugated goat anti-mouse antibodies (Dako, 1 µg/ml in PBST). After a final wash step (3× 15 min), the membrane was incubated for 1 min in ECL detection reagent (Amersham, Little Chalfont), and exposed for 1 min to ECL film (Amersham). After the film was developed, the membrane was stripped with 8 M urea/1% SDS/0.1 M β-mercaptoethanol for 30 min, and finally with ethanol/water/acetic acid (5:5:1) for 30 min. The above procedure was then repeated except for the RPA incubation.

Peptide analysis

The following 20 amino acid peptides were used linked via SGSG residues at the N-terminus to biotin (Chiron Mimotopes, Clayton, Australia). Each peptide was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 5 mg/mg. p21, KRRQTSMTDFYHSKRRLIFS; p21-A, KRRATSMDFYHSKRRLIFS; UDgH, MIGQKTLYSFFSPSPARKRH; UDgH-A, MIGAKTLYSFFSPSPARKRH; UDgM, MIGQKTLYSFFSPSPPTGKRT; UDgM-A, MIGAKTLYSFFSPSPPTGKRT; UDgY, KRKQTTIEDFFGTTKSTNEA; UDgY-A, KRKATTIEDFFGTTKSTNEA; unrelated, PESVELKWSEPNNEELIKFM.

Peptide pull-down experiments

Approximately 2.5 µg of each peptide was incubated with 10 µl of streptavidin-agarose beads (Sigma, St Louis, MO) in PBS for 1 h at room temperature, the beads were then washed extensively in PBS and recovered each time by centrifugation. A 20 µl aliquot of either HeLa cell extract or *S.pombe* protein extract diluted in PBS to a final concentration of 1 mg/ml was added to the washed beads and incubated with the beads on ice for 1 h. The beads were washed extensively in PBS containing 0.05% Tween-20, and bound proteins removed by boiling in SDS-PAGE loading buffer for 5 min. Proteins were separated on 15% SDS-PAGE and electrophoretically transferred to PVDF membrane (Amersham). The membranes were blocked in PBS containing 2% skimmed milk for 30 min, then incubated for 1 h with the monoclonal anti-PCNA antibody PC10 diluted 1:1000 in 2% skimmed milk-PBS. This antibody recognizes PCNA from a range of species including human and *S.pombe* (Waseem and Lane, 1990). After washing, blots were incubated with secondary HRP-conjugated rabbit anti-mouse antibodies (Dako) diluted 1:1000 in 2% skimmed milk-PBS for 1 h, followed by washing in PBS/0.05% Tween-20. Bound antibody was visualized using the ECL system according to the manufacturer's instructions (Amersham).

Peptide competition experiments

In competition experiments, non-biotinylated peptides were added to the diluted cell extracts before incubation with the immobilized, biotinylated peptides. The peptides used were a p21-derived peptide (KRRQTSMTDFYHSKRRLIFS) or an unrelated control peptide (KPVRLPISQAIKCAP) added from a stock dissolved in DMSO at 20 mg/ml to a final concentration of 1 mg/ml. In each case, a control reaction was carried out in which an equivalent amount of DMSO was added to control for the effects of the solvent.

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