Perpetuation rather than repair of a DNA lesion.

Richard F. Pollock[1,*], Javier M. Di Noia[2] and Michael S. Neuberger[2]

[1]Trinity College, Cambridge, CB2 1TQ, UK
[2]Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK
[*]Corresponding author

Somatic hypermutation occurs at both C•G and A•T pairs in immunoglobulin genes in B lymphocytes. Phase I somatic hypermutation, involving mutation at C•G pairs, occurs via Activation-Induced Deaminase (AID)-catalysed deamination of deoxycytosine (C) to deoxyuridine (U). The deamination results in the formation of a U•G lesion, after which the cell may either replicate or the uracil-DNA glycosylase (UNG) may excise the uracil to give an abasic site. If the cell replicates, the replication fork runs over the deoxyuridine, giving rise to C→T and G→A transition mutations (Pyrimidine to Pyrimidine and Purine to Purine). Of the resulting daughter cells, one will have a wild-type C•G pair and the other will have a U•A pair (which will give rise to T•A pairs in future generations) – this is phase IA hypermutation. If, rather than replicating the DNA, UNG activates the base excision repair (BER) pathway, the uracil will be excised leaving an abasic site. At this point, either the cell may replicate or the apyrimidinic endonuclease (APE) will introduce a nick into the backbone, resulting in polymerase-mediated repair of the lesion using deoxyguanosine as a template. If replication occurs at this point, the lesion undergoes phase IB hypermutation which, due to the non-instructional nature of the lesion, may introduce either transition or transversion mutations (i.e. any nucleobase may be inserted into the daughter DNA strand). Here we report that the decision to replicate DNA rather than repair via the BER pathway may be due to a temporal constraint imposed by the interaction of UNG with PCNA, a component of the replication apparatus.

INTRODUCTION

In somatic cells that do not undergo hypermutation, the presence of a foreign deoxyuracil base (introduced, for example, by spontaneous deamination of cytosine) is recognised by the uracil-DNA glycosylase (UNG). The uracil is flipped out of the helix into the active site of UNG to leave the site abasic, as illustrated in Figure 1. However, in the immunoglobulin genes, there is targeted deamination of cytosine, catalysed by Activation-Induced Deaminase (AID). The resulting lesion is not found to be repaired, but rather a point mutation at the site of the lesion is found to be perpetuated in subsequent generations of the cell. One proposed mechanism for the perpetuation of the point mutant rather than its repair is the replication of the cell's DNA immediately after either deamination (phase IA) or base excision (phase IB). In such a model, no DNA repair enzymes "downstream" of uracil excision have the chance to repair the lesion before either a standard replicative polymerase or a *trans*-lesion polymerase replicate the DNA.

If the proposed model is correct, the enzyme responsible for either deaminating (AID) or excising (UNG) the base would be expected to show some association with the replication fork. An excellent candidate for such an interaction is the Proliferating Cell Nuclear Antigen (PCNA), which forms a toroidal-shaped trimer around both the leading and lagging strands of DNA during replication. Loading of PCNA onto the DNA (at the primer-template junction) requires ATP hydrolysis by the accessory clamp loader replication factor C, which then dissociates once the PCNA is fully loaded. PCNA is an essential component of the replication apparatus as it is a necessary polymerase processivity factor. Any interaction between PCNA and either AID or UNG would be broadly supportive of the model, as it could be inferred that such a PCNA:AID or PCNA:UNG complex is responsible for either pre- or immediately post-replicative deamination or base excision, depending upon the orientation of the interaction with PCNA. Indeed, a PCNA:UNG interaction has previously been demonstrated in yeast two-hybrid and immunoprecipitation methods.

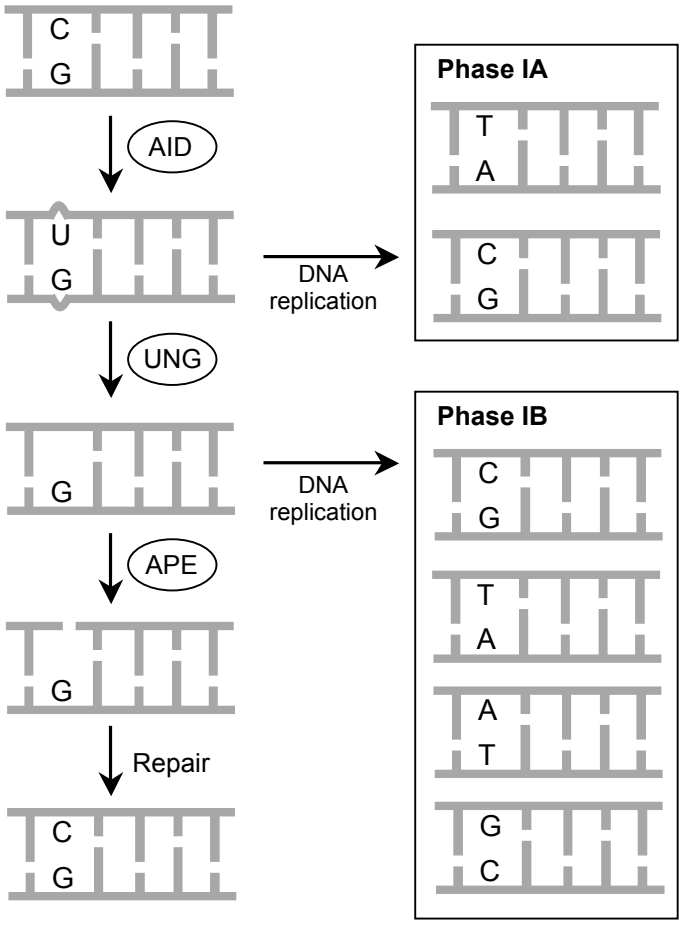

Figure 1 | **Somatic hypermutation mediated by AID-catalysed cytosine deamination.** Adapted from Neuberger *et al.* Nature Immunology Reviews 5 171-178 (Feb 2005)

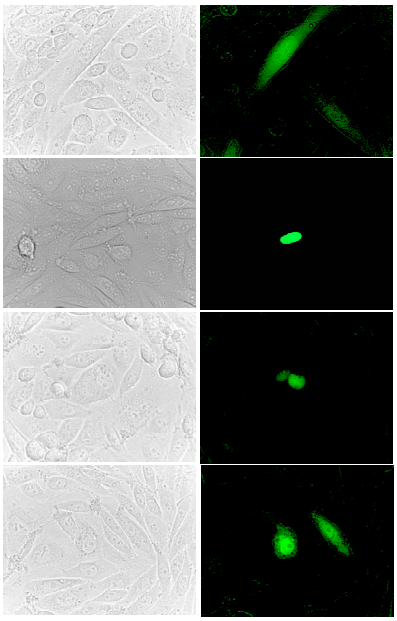

Figure 2 | **Subcellular localisation of UNG and UNG double point mutants in the PCNA-binding motif.** The PCNA binding motif and nuclear localisation signal overlap at the N-terminus of UNG. The 44 N-terminal residues unique to the UNG2 isoform are essential but not sufficient for nuclear localisation and residues 4-11 are both essential and sufficient for interaction with PCNA. Disruption of the PCNA binding motif may therefore affect nuclear localisation. In this figure, both images in each pair were produced by z-acquisition at 40x magnification using standard light microscopy on the left and with UV illumination on the right. The same cells are visible in each pair of images. The Q4AL7R mutant appears to disrupt localisation specifically to the nucleus, but closer inspection reveals that the mutant still shows a preference for nuclear localisation.

RESULTS

Molecular interactions between PCNA and UNG

The *UNG* gene encodes both mitochondrial (UNG1) and nuclear (UNG2) forms of the glycosylase using different promoters and alternate splicing. The well-characterised catalytic domain of the two isoforms is conserved while each has a unique N-terminal subcellular sorting motif giving rise to the different localisations of the splice variants. From hereon, UNG will refer solely to the UNG2 isoform, which was the only isoform used in these assays.

Many proteins that interact with PCNA contain the conserved PCNA binding motif $QX_2(L/M/I)X_2(F/Y/H)(F/Y)$. A sequence matching this consensus has been found at the N-terminus of both mUNG and hUNG: ^{4}QKTLYSFF^{11}. To test for PCNA:UNG and PCNA:AID interactions, a mammalian two-hybrid system was used in Chinese Hamster Ovary (CHO) cells. The first results from this system are reported in Table 1 and illustrated in Figure 3. Compared to the negative, PCNA in a VP16 vector with an empty GAL4 vector, there was a 384 fold increase when testing the UNG:PCNA interaction. The interaction was shown to be approximately three times stronger than the positive control of PCNA trimerisation.

Table 1 | **Interaction between various VP16 and GAL4 fusions as determined by luciferase expression from a GAL4 promoter.**

VP16	GAL4	RLUs (x1000)	x-fold increase over negative
hPCNA	hPCNA	48	124.8
mUNG	hPCNA	148	386.4
hAIDΔC	E47	12	31.2
hAID	E47	1.6	4.3
hPCNA	-	0.4	1.0

As an aside from the primary experiment, AID ΔC, lacking a 15 amino acid C-terminus nuclear export signal, was shown to have an increased (~8 fold) interaction with the basic helix-loop-helix (bHLH) transcription factor E47 over AID. E47 is a key regulator of B lymphocytes, binding to E-boxes in regulatory regions of B lymphocyte-specific genes, particularly enhancers in the immunoglobulin loci and promoters of mb-1, λ5 and RAG-1. E47 is therefore crucial in regulating the expression of the surrogate light chain Ig, promoting cell survival in early pre-B cells and initiation of Ig rearrangements. While any further investigation of gene rearrangement was beyond the scope of this project, E47 has also been proposed as playing a role in somatic mutation in mature B cells (Storb, U. April 2001). The discovery that AID ΔC (i.e. AID localised in the nucleus) has some interaction with the E47 may imply that it does indeed play a role in phase IA somatic mutation.

Subcellular localistion of UNG and UNG mutants

The subcellular localisation of UNG and two mutants (F9AF10A and Q4AL7R – both double mutants in the PCNA binding motif) was assayed in CHO cells using a simple EGFP fusion (such that GFP was fused at the C-terminus) and observed using a standard light microscope at 40x magnification. The results are illustrated in Figure 2. The Q4AL7R mutant appeared to disrupt the exclusive nuclear localisation of the UNG, although the fusion protein still showed preferential localisation in the nucleus, to a high enough concentration to perform the mammalian two-hybrid. The F9AF10A double mutant had no visible effect on subcellular localisation of the fusion protein.

Disruption of the UNG:PCNA interaction

The aforementioned UNG double point mutants, along with a single L7R point mutant, were produced by PCR using a primer mismatched at the sites of interest and tested in the mammalian two-hybrid for interaction with PCNA (Figure 4). All mutations were located in the 4-11 PCNA binding

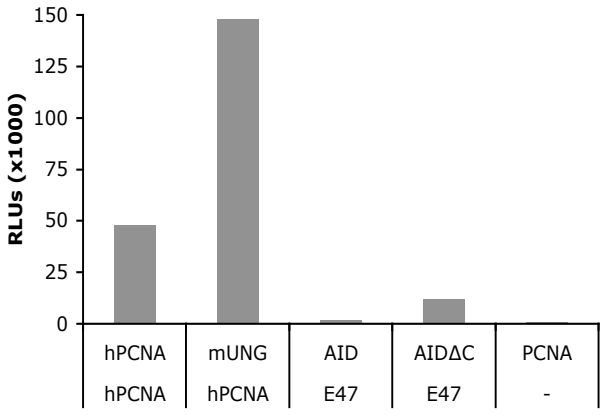

Figure 3 | **Interaction between VP16 and GAL4 fusions in CHO cells.** VP16 fusions are listed above GAL4 fusions on the x-axis. Relative Luminescence Units were calculated and normalised for transfection efficiency as described in Materials and Methods.

Table 2 | **Interaction between PCNA and UNG mutants, assayed by luciferase expression from a GAL4 promoter.**

VP16	GAL4	RLUs (x1000)	x-fold increase over negative
hPCNA	hPCNA	42.4	59.1
mUNG	hPCNA	41.2	57.4
F9AF10A	hPCNA	3.1	4.3
Q4AL7R	hPCNA	7.7	10.7
L7R	hPCNA	2.4	3.4
hPCNA	-	0.7	1.0

```
mUNG2    MIGQKTLYSFFSPTPTGKRTTRSPEPVPGSGVAAEIGGDAVASPA

L7R      MIGQKTRYSFFSPTPTGKRTTRSPEPVPGSGVAAEIGGDAVASPA
F9AF10A  MIGQKTLYSAASPTPTGKRTTRSPEPVPGSGVAAEIGGDAVASPA
Q4AL7R   MIGAKTRYSFFSPTPTGKRTTRSPEPVPGSGVAAEIGGDAVASPA
```

Figure 4 | **UNG mutants.** N-terminus (1-45) of mUNG2 and mutants.

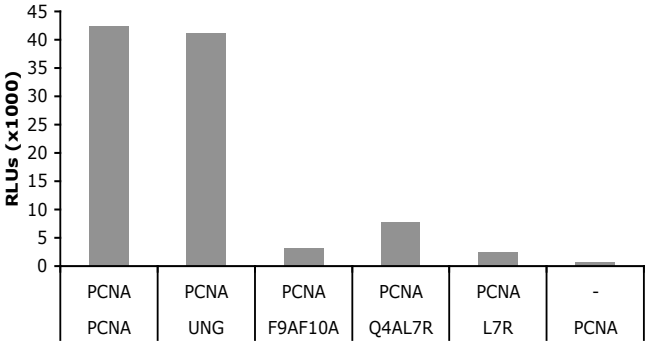

Figure 5 | **Interaction between PCNA and UNG mutants in CHO cells.** VP16 fusions are listed above GAL4 fusions on the x-axis. Relative Luminescence Units were calculated and normalised for transfection efficiency as described in Materials and Methods.

motif. The results from these two-hybrid assays are reported in Table 2 and illustrated in Figure 5. Relative to the wild type UNG:PCNA interaction, all of the UNG mutants had a deleterious effect the interaction with PCNA, decreasing the interaction by between 5.3 and 17 times. The L7R mutant disrupted the PCNA:UNG interaction more than the Q4AL7R double mutant with the possible implication that the Q4A point mutation in fact increases the strength of binding to PCNA.

DISCUSSION

From the two-hybrid assay, it is clear that there is a strong interaction between UNG and PCNA in CHO cells. Furthermore, this interaction can be disrupted by introducing mutations into UNG's PCNA binding motif. Due to time constraints and problems obtaining the correct vectors for the assay, the PCNA:AID interaction was not tested but, now the assay system is working, it would be relatively straightforward to test for this interaction.

The light microscopy on the UNG mutant:GFP fusions was instructive, revealing that only the Q4AL7R UNG mutant noticably disrupted the normal nuclear localisation of UNG. However, this localisation assay should be repeated using confocal microscopy and more samples for higher resolution and to increase reliability of the results.

Going forward with the results from the two-hybrid system, the assay should be repeated many more times to establish the background of the system and the reproducibility of the results. Furthermore, reproduction of the assay would reveal the significance of the interactions between PCNA and the various UNG mutants. Looking further ahead, tests for restoration of gene conversion and transversions should be performed in UNG-knockout DT40 cells transfected with the various UNG mutants. Additionally, attempts should be made to restore immunoglobulin class switching in UNG-deficient B lymphocytes by retroviral infection with the UNG mutants.

MATERIALS AND METHODS

Mammalian two-hybrid methods

CHO cells, grown in culture for use in the two-hybrid system, were transfected with two plasmid vectors containing luciferase: pRL-CMV (*Renilla* luciferase under a constitutive promoter) and pG5luc (*Photinus pyralsis* luciferase under control of the GAL4 promoter) (Promega DLR Assay). A further two vectors, one (pVP16 or pMVN) containing fusions of UNG, AID, AID ΔC, E47, REV1 and PCNA with the VP16 activation domain and the other (pM or pAM) containing fusions of UNG, AID, AID ΔC, E47, PCNA with the GAL4 DNA-binding domain were transfected in various combinations. One GAL4 and one VP16 fusion was present in each assay. CHO cells were plated at 100,000 ml^{-1} in a 24-well plate 24 hours before transfection. Transfection was performed with 2 μl Lipofectamine per well with a 5 hour incubation with serum-free F12-K medium (Kaign's modification) (refer to Invitrogen manual). A total of 0.25 μg of DNA was transfected into each well: 10% PRL-CMV, 30% pG5luc, 30% VP16 fusion and 30% GAL4 fusion. The cells were left for 48 hours between transfection and measurement with the Promega Dual Luciferase Assay system in which 20 μl of the cell lysate was placed into a 96-well luminometer plate with a program set to dispense 50 μl of the luciferase assay reagent LARII, measure *Photinus* luminescence for 10 s, dispense 50 μl Stop & Glo™ reagent and measure constitutive *Renilla* luminescence for 10s. Relative Luminescence Units (RLUs) were then calculated by dividing *Photinus* luminescence by *Renilla* luminescence to normalise for transfection efficiency.

PCNA:PCNA and PCNA:REV1 were used as positive controls as follows: pVP16 PCNA with pM PCNA and pVP16 REV1 with pM PCNA. Negative controls consisted of pVP16 PCNA with pVP16 UNG, pVP16 PCNA with empty pM vector and pM PCNA with empty pVP16 vector.

Bright-field light microscopy for observation of subcellular localisation of GFP:UNG-mutant fusions

To determine the subcellular localisation of the various GFP:UNG mutant fusions, CHO cells were plated at 100,000 ml^{-1} in 24-well plates. The cells were cultured in F12-K (Kaign's modification) with 10% serum before being transfected using a total DNA mass of 0.25 μg (pEGFPC1 with UNG mutant inserts) using 0.8 μl FuGENE per well. Following transfection, cells were left for 48 hours before observation using a bright field microscope with a 40x objective. For GFP visulisation, a UV light source was used in place of the built-in visibile light source. In each case, the same field of vision was recorded with both UV and visiblie light using a compuer-attached camera recording to 9 megapixel TIFF.