Human mitochondrial uracil-DNA glycosylase preform (UNG1) is processed to two forms one of which is resistant to inhibition by AP sites

Sangeeta Bharati, Hans E. Krokan, Lena Kristiansen, Marit Otterlei and Geir Slupphaug*

Institute for Cancer Research and Molecular Biology, Norwegian University of Science and Technology, N-7005 Trondheim, Norway

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ABSTRACT

The preform of human mitochondrial uracil-DNA glycosylase (UNG1) contains 35 N-terminal residues required for mitochondrial targeting. We have examined processing of human UNG1 expressed in insect cells and processing in vitro by human mitochondrial extracts. In insect cells we detected a major processed form lacking 29 of the 35 unique N-terminal residues (UNG1Δ29, 31 kDa) and two minor forms lacking the 75 and 77 N-terminal residues, respectively (UNG1Δ75 and UNG1Δ77, 26 kDa). Purified UNG1Δ29 was effectively cleaved in vitro to a fully active 26 kDa form by human mitochondrial extracts. Furthermore, endogenous forms of 31 and 26 kDa were also observed in HeLa mitochondrial extracts. The sequences at the cleavage sites, as identified by peptide sequencing, were compatible with the known specificity of mitochondrial processing peptidase (MPP). However, in vitro cleavage of UNG1Δ29 by mitochondrial extracts did not require divalent cations and was stimulated by EDTA, indicating the involvement of a processing peptidase distinct from MPP at the second site. Interestingly, while UNG1Δ29 generally has the typical properties reported for other uracil-DNA glycosylases, it is not inhibited by apurinic/apyrimidinic sites. Our results indicate that the preform of human mitochondrial uracil-DNA glycosylase is processed to distinctly different forms lacking 29 or 75/77 N-terminal residues, respectively.

INTRODUCTION

Uracil-DNA glycosylases (UDG) catalyse the first step in the base excision repair (BER) pathway for removal of uracil from DNA. Uracil in DNA results from either deamination of cytosine, a premutagenic lesion (1), or incorporation of dUMP instead of dTMP during replication (2). Genes or cDNA sequences for UDGs from different organisms predict sizes ranging from 204 to 359 amino acid residues. Crystal structures of the HSV-1 (3) and the human (4) C-terminal regions of ~220 residues are very similar and contain both the DNA-binding and catalytic domains (3). Catalysis proceeds via the insertion of a conserved leucine into the DNA minor groove following expulsion of uracil into the buried catalytic pocket, concomitant with compression of flanking DNA phosphates and surrounding amino acid residues to form a productive complex (5). N-terminal sequences of UDGs are not required for catalytic activity and vary widely both in length and amino acid composition. They are considerably longer in the eukaryotic and herpesviral UDGs as compared with the bacterial, mycoplasma and poxviral UDGs, in agreement with their involvement in subcellular localization in mammalian cells (6,7). The human UNG gene was the first mammalian gene demonstrated to encode both nuclear (UNG2) and mitochondrial (UNG1) isoforms of an enzyme (6), by a mechanism involving transcription from two different UNG promoters and alternative splicing (7). The mRNAs for UNG1 and UNG2 encode 35 and 44 unique N-terminal residues that are required for mitochondrial and nuclear translocation, respectively, whereas the 269 residues downstream of the unique sequences are common to the two isoforms (7).

Purification of UNG proteins from various cells and subcellular fractions have resulted in active enzyme species that differ in size as well as in some biochemical properties (6,8-11). Recently, purification of human UNG protein from HeLa cells in the presence of a cocktail of protease inhibitors resulted in an enzyme species of 35–37 kDa, which is close to the size predicted from the open reading frame for UNG2 and UNG1 mRNAs (12). A part of the N-terminal region in UNG located C-terminal to the unique sequences has been demonstrated to bind replication protein A (13). Thus, N-terminal regions in UNG proteins in addition to having a role in subcellular targeting may also have functions that are not yet elucidated. In the present work we present evidence that the mitochondrial preprotein UNG1 is processed to yield two catalytically active forms of 31 and 26 kDa, respectively. In addition we show that the 31 kDa form has certain unique properties not described for other uracil-DNA glycosylases, most notably the resistance to the inhibitory effect of AP sites observed with other UDGs.

*To whom correspondence should be addressed. Tel: +47 73598693; Fax: +47 73598801; Email: geir.slupphaug@medisin.ntnu.no
MATERIALS AND METHODS

Materials

The BacPAK Baculovirus Expression System kit was from Clontech Laboratories (Palo Alto, CA). Protease K, RNase A and SeaPlaque agarose were from Sigma (St Louis, MO). The protease inhibitors and DIG glycan/protein double labelling kit were from Boehringer-Mannheim (Germany). Anti-phosphoprotein antibodies were from Zymed Laboratories (San Francisco, CA). Restriction enzymes, DNA-modifying enzymes and linkers were from New England Biolabs (Beverly, MA) and Promega (Madison, WI). Media components for bacterial culture were from Difco (Detroit, MI) and media for insect cell culture were from Gibco BRL (Gaithersburg, MD). Chromatographic matrices were from Pharmacia Biotech (Upssala, Sweden).

Plasmid constructions, co-transfection and generation of recombinant virus

In brief, the pSELECT system (Promega) was used to introduce a NdeI site at the ATG start codon (position 110) in UNG15 (14), containing the complete UNG1 open reading frame. After blunting of the NdeI site, XhoI linkers were added directly upstream of the ATG codon. The XhoI–Hpal fragment of UNG15 containing its stop signal was cloned into the XhoI and SmaI sites in the polylinker of the pBacPAC8 baculovirus expression vector (Clontech). The resultant construct was named pBacUNG1 (6.5 kb) and correct in-frame sequence was confirmed by DNA sequencing. Spodoptera frugiperda S21 cells were cultured and infected with recombinant virus essentially as described (15). Lipofectin-mediated co-transfection of pBacUNG and AcMNPV or BacPAK6 viral DNA into S21 cells was performed according to the manufacturer’s protocol (Clontech). The recombinant viruses were isolated 72 h post-infection by several rounds of plaque purification (15) and screened by activity measurements and Southern analysis using an UNG-specific probe. The recombinant virus BacUNG10 was selected for scale-up experiments.

Purification of recombinant UNG

S21 cells were cultured in suspension cultures, infected with the recombinant baculovirus at a multiplicity of infection of 20 and harvested 36 h post-infection. All subsequent steps were performed at 4°C. The frozen cell pellet (62 g) from 71 culture was thawed and resuspended in 200 ml of lysis buffer containing 50 mM Tris–acetate (pH 7), 10 mM NaCl, 1 mM DTT and protease inhibitors (all buffers used during protein purification were degassed and contained 0.5 mM PMSF, 1 µg/ml each of pepstatin A and leupeptin and 2 µg/ml of aprostatin). Cells were lysed by sonication and complete cell disintegration was verified by phase-contrast microscopy. UNG proteins were purified essentially as described (16) except that nucleic acid precipitation using protamine sulphate was omitted. Fractions from each chromatographic step were analysed by SDS–PAGE and western analysis using the polyclonal antibody PU101 directed against the catalytic 26 kDa domain (16). Purified proteins were thoroughly dialysed against 50 mM NH4HCO3 and N-terminally sequenced using an Applied Biosystems 477A Sequenator.

Subcellular fractionation of HeLa cells

In brief, 2 × 107 logarithmically growing HeLa cells were harvested by scraping and washed twice in ice-cold PBS, resuspended in 5 ml hypotonic buffer (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl2) and lysed by 15 strokes in a 7 ml Dounce homogenizer. Nuclei were pelleted by centrifugation at 500 g for 2 min and further purified in a 30/55% OptiPrep (Nycoderm Pharma, Norway) step gradient according to the manufacturer’s recommendations. Finally, the nuclei were incubated in 0.5 ml PBS, 0.2% Triton X-100 for 30 min on ice, adjusted to a final protein concentration of 1 mg/ml in the same buffer, snap frozen in liquid N2 and stored at −70°C. For fractionation of mitochondria, 108 HeLa cells were harvested as above, resuspended in 6 ml diluent B (8% sucrose, 1 mM EDTA, 20 mM Tricine–NaOH, pH 7.8) and lysed by 30 strokes in a 7 ml Dounce homogenizer. The homogenate was centrifuged at 1000 g for 10 min, the pellet resuspended in 5 ml diluent B and recentrifuged as above and the two post-nuclear supernatants (PNS) combined. The PNS was further centrifuged at 3000 g for 10 min to pellet the heavy mitochondrial fraction (HMF) and the supernatant recentrifuged at 17 000 g for 15 min to pellet the light mitochondrial fraction (LMF). The LMF was further fractionated in an OptiPrep continuous density gradient (starting concentration 17%) at 270 000 g for 3 h. All fractions, including the LMF supernatant (cytosol/microsomal fraction) were immediately snap frozen in liquid N2 and stored at −70°C.

Assays

Unless otherwise specified, UDG activity was measured in 20 µl of assay mixture under the standard conditions essentially as described (16). BioRad protein assay was used to measure protein concentrations using BSA as standard. Km, Vmax and uracil inhibition were determined in the presence of 0.5–10 µM [3H]dUMP-containing DNA (concentration referring to uracil). Single-stranded (ss) substrate was prepared by heating the double-stranded (ds) substrate at 100°C for 10 min and then immediately cooling on ice. The concentrations of the enzyme protein were 0.1 and 0.3 ng/20 µl assay mixture for ssDNA and dsDNA, respectively, and the NaCl concentrations were 10, 30 and 60 mM. Inhibition by uracil was studied in the presence of 2 and 5 mM uracil at each of the above NaCl concentrations. Marker enzymes for the following organelles were assayed essentially as described; cytochrome c oxidase (mitochondrial marker) (8), acid phosphatase (lysosomes) (17) and catalase (peroxisomes) (18). Sequence specificity of uracil excision was assayed essentially as described (19) and binding of UNG to ssDNA or dsDNA matrices were as described (4) except that larger matrix volumes (0.5 × 5 cm) were used. Post-translational glycosylation and phosphorylation were assayed using a DIG glycan/protein double labelling kit and anti-phosphoprotein antibodies, respectively, according to the recommended protocols. The effect of HAP1 (a gift from I. D. Hickson, Oxford, UK) on uracil excision was assayed by including varying concentrations of HAP1 (0–250 times molar excess with respect to UNG) in standard UDG assays. For a time curve experiment, similar assays were performed for specific lengths of time (0–60 min) using UNG only (50 pmol) or UNG (50 pmol) and a 25× molar excess of HAP1. The effect of AP sites was studied by including the different specified concentrations of double-stranded oligonucleotides containing AP
Expression of human UNG in insect cells

Infection of Sf21 insect cells with BacUNG10 resulted in a new protein that was easily detected 24 h post-infection when cells were directly lysed in denaturing buffer and subjected to SDS–PAGE and western analysis (Fig. 1A). UDG activity was not detected in uninfected controls and the anti-UNG antibody used in western analyses did not cross-react with insect proteins. UNG proteins migrate more slowly than expected from their calculated Mr  of full-length UNG1 is 33.9 kDa, which would be expected to migrate as a band of an apparent Mr of 35–36 kDa. No UNG protein of this size was observed 24 h post-infection. In fact, the major recombinant UNG1 species migrated corresponding to an apparent Mr of 32 kDa. As demonstrated below, this band represents a protein of 31 kDa. Increasing the time after infection resulted in a marked accumulation of this 31 kDa species. In addition, several new bands of lower Mr were detected, apparently representing degradation products of UNG1. Thirty-six hours post-infection, an additional faint band of ∼36 kDa was detected which most likely represents the full-length UNG1 protein. The amount of the putative full-length species decreased upon further incubation, indicating that in vivo cleavage of full-length UNG1 occurs effectively in the insect cells. Figure 1B shows the UDG activity of cell-free extracts at different time points after infection. A sharp rise in specific activity was observed between 24 and 36 h, concomitant with a marked increase in the 31 kDa species and the appearance of a new band corresponding to a 26 kDa UNG species (Fig. 1A). At 48 h a slight decrease in the 31 kDa band was observed concomitant with an increase in the 26 kDa band and a further increase in specific activity. The 26 kDa band has an electrophoretic mobility similar to UNG1Δ84 (16), which may explain the observed increase in specific activity since UNG1Δ84 was shown to exhibit the maximal UDG activity of a series of N-terminally deleted UNG1 mutants expressed in rabbit reticulocyte lysates. Mutational analysis furthermore demonstrated that the C-terminus of UNG1Δ84 was essential for catalytic activity (16). The present results thus suggest that full-length human UNG1 is efficiently N-terminally cleaved in insect cells lacking endogenous UDG, yielding two enzymatically active forms of 31 and 26 kDa, respectively.

Purification of UNG1 from insect cells

In an attempt to purify UNG1-derived proteins, a 7 l suspension culture of baculovirus-infected Sf21 cells was harvested 36 h post-infection. Western analysis demonstrated that only trace amounts of the putative full-length protein were present after lysis in non-denaturing buffer (data not shown). Addition of a protease inhibitor cocktail containing PMSF, pepstatin A, leupeptin and aprotinin prior to cell homogenization did not improve the yield of full-length UNG1, supporting that proteolysis occurs in vivo. Samples from the various purification steps were analysed by SDS–PAGE and silver staining (Fig. 2) and the 31 kDa protein was apparently homogeneous after the final MonoS purification step. The identities of the N-terminal amino acids of the purified enzyme were determined by automated Edman degradation. This yielded the sequence CGDHLQAIPAKK, thus representing an enzymatically active species lacking the 29 N-terminal amino acids (UNG1Δ29) of UNG1 and having a calculated molecular mass of 31 kDa (Fig. 3). A 26 kDa UNG fraction was also purified essentially as above and N-terminal sequencing showed that this
Human mitochondria contain two processed forms of UNG1, one of which is not formed by MPP

To investigate whether the observed UNG1 processing in insect cells also occurs in human cells, HeLa mitochondria were isolated by OptiPrep density gradient centrifugation and subjected to SDS–PAGE and western analysis using a polyclonal antibody directed towards the common catalytic domain of UNG1 and UNG2. As shown in Figure 4, two bands were identified representing 31 and 26 kDa UNG species, of which the 31 kDa species was quantitatively dominant. In addition, a band was observed at ~36 kDa, most likely representing unprocessed UNG1. A weak band of >36 kDa was also observed, which most likely represents unspecific staining, since no human UNG proteins of this size are known. This indicated that the mitochondrial cleavage of UNG1 is similar in insects and humans. Furthermore, since Sf21 cells do not appear to express UDG activity, the cleavage factors are unlikely to be specific for UNG proteins.

To investigate whether the cleavage factor(s) was specific for mitochondria, various HeLa subcellular fractions were isolated and purified recombinant UNG1Δ29 fractions were incubated in the presence of various fractions after refractionation of the light mitochondrial fraction; (lower) cytochrome c oxidase activity (filled circles) and endogenous UDG activity (filled triangles) in individual fractions.

To investigate whether the cleavage factors were specific for mitochondria, various HeLa subcellular fractions were isolated and purified recombinant UNG1Δ29 incubated in the presence of various fractions after refractionation of the light mitochondrial fraction, yielding a 26 kDa UNG species (Fig. 5A) concomitant with a 35% increase in UDG activity (data not shown). Detectable cleavage, although very weak, was also observed with the PNS and nuclear extract, while the cytosol displayed no detectable proteolytic activity towards UNG1Δ29. It is possible that cleavage associated with PNS and nuclear fractions is caused by a small contamination by mitochondria or mitochondrial fragments, although no significant cytochrome c oxidase activity was associated with these fractions (data not shown).

Density gradient fractionation of the LMF demonstrated that the UNG1Δ29 cleaving activity and the cytochrome c oxidase activity co-eluted. The same fractions also contained the major fraction of endogenous UNG activity in the LMF (Fig. 5B), although a significant portion of the endogenous UNG activity could not be assigned to a specific organelle (fractions 2–3). The latter activity may be due to mitochondrial leakage during fractionation or release of loosely attached UNG from the mitochondrial surface. Contamination of the LMF by lysosomal or peroxisomal proteases was unlikely, since no marker enzyme activity specific for these organelles could be detected. Although the N-terminus of this 26 kDa UNG could not be determined by amino acid sequencing, previous isolation of UNG1Δ77 from
Since MPP generally cleaves close to the signal sequence. Surprisingly, no cleavage of purified UNG1Δ29 was observed after incubation with cell-free extracts or purified mitochondria from untransfected insect cells in the absence of protease inhibitors (data not shown). It thus remains to be determined if cleavage of UNG1Δ29 by human and insect cells occurs by similar proteases.

To investigate the general susceptibility of UNG1Δ29 to proteolytic cleavage, the enzyme was subjected to proteinase K digestion (Fig. 7A and B). After 1.5 h incubation the protein was apparently cleaved to a single species of the same apparent M₈ (26 kDa) as observed after cleavage by mitochondrial extracts. After 6 h incubation, the amount of this species was significantly reduced as compared with the controls, indicating further slow proteolytic breakdown of the 26 kDa species. Slow proteolysis was also observed with UNG1Δ77 and after 6 h the intensity of the 26 kDa band was similar in both reactions treated with proteinase K (Fig. 7A). Cleavage of UNG1Δ29 was accompanied by a 2.4-fold increase of the enzymatic activity after 1.5 h (Fig 7B) and after 6 h the specific molar activity was similar to that of UNG1Δ29 treated with proteinase K in parallel reactions. These results may indicate that the N-terminal presequence of UNG1 is separated from the compact and protease-resistant catalytic domain by a linker region susceptible to general proteolysis. The specificity of the observed Δ75/Δ77 cleavage thus remains to be elucidated.

**Catalytic properties of UNG1Δ29**

The presence of a 31 kDa UNG in human mitochondria resembling UNG1Δ29 and the fact that this species is enzymatically highly active justified further biochemical analysis of UNG1Δ29. The kinetic constants of UNG1Δ29 measured at different NaCl concentrations are given in Table 1. A similar salt optimum (60 mM) was observed for UNG1Δ29 as for UNG1Δ77 purified...
from human placenta (9), but \( k_{cat}/K_m \) of UNG1Δ29 was \(~6\) fold lower. Analysis of possible post-translational modifications in UNG1Δ29 demonstrated that the protein was neither glycosylated nor phosphorylated (data not shown). The specific activity of UNG1Δ29 using ssDNA as substrate was \(~3\) fold higher than that obtained with dsDNA substrate under standard assay conditions, similar to other UNG enzymes reported (9,16,22). Furthermore, the sequence context preference for uracil excision was essentially identical to that of UNG1Δ84 (16; data not shown).

### Table 1. Kinetic analysis of recombinant UNG1Δ29

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<tr>
<th>NaCl (mM)</th>
<th>( k_{cat} ) (µM)</th>
<th>( k_{cat} ) (µM s(^{-1}))</th>
<th>( k_{cat}/K_m ) (µM(^{-1}) s(^{-1}))</th>
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<tbody>
<tr>
<td>dsDNA</td>
<td>1.0</td>
<td>1.3</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>ssDNA</td>
<td>ND</td>
<td>0.5</td>
<td>0.9</td>
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<td></td>
<td>10</td>
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\( k_{cat}/K_m \) values were not determined (ND).

### DISCUSSION

Using a baculovirus expression system, we have produced a 31 kDa recombinant form of human UNG1 (UNG1Δ29) that lacks 29 of the 35 N-terminal amino acids unique to UNG1, but which contains the complete common domain of UNG1 and UNG2. This form represents an enzymatically highly active protein that is apparently also present in human mitochondria in addition to the fully processed 26 kDa core catalytic domain. Cleavage resulting in the 26 kDa UNG1 takes place within the N-terminal sequence common to UNG1 and nuclear UNG2. This most likely explains the different results from various laboratories with regard to sizes and properties of UNG proteins purified from different mammalian cells, since leakage of mitochondrial proteases during cell fractionation could result in cleavage of UNG2, thus producing an enzyme species identical to the 26 kDa UNG1.

Whether the 31 or the 26 kDa form represents the mature functional mitochondrial UNG remains unclear. Alternatively, both forms are present and serve distinct functions in mitochondrial uracil repair. Quantitatively, the 31 kDa form dominates both in the insect cells and isolated HeLa mitochondria, indicating that UNG1Δ29 is a functional mitochondrial enzyme. The presence of a putative amphiphilic helix at UNG1 positions 11–29, the cleavage immediately downstream of this region and the homology of the cleavage site to known MPP substrates further suggest that UNG1 is translated via the major mitochondrial import machinery (reviewed in 26) and cleaved by MPP to yield UNG1Δ29. This is also supported by the apparently effective \( in vivo \) formation of UNG1Δ29 in insect cells lacking endogenous UDG. Further, octapeptidyl removal by the mitochondrial intermediate peptidase (MIP) was ruled out, since no species of such an intermediate size were observed and no homology to known MIP substrates was found. The specificity of cleavage in the \( \Delta 77 \) region yet remains to be determined. The fact that UNG1Δ77 is present in human placental extracts (9), in the recombinant insect cells and apparently intact HeLa mitochondria may indicate a protease of similar specificity. However, since UNG is normally not present in the insect cells, this protease is likely to have a more generalized function. This is also supported by the apparent
susceptibility to general proteolytic cleavage in the Δ77 region as demonstrated by proteinase K cleavage.

Two enzymatically active UDGs have also been demonstrated in rat liver mitochondria, one of which is apparently formed by proteolysis of the other (11). This may support the view that mammalian mitochondria contain two distinct functional UDGs. Interestingly, however, both the rat mitochondrial UDGs were inhibited by AP sites (11) and may reflect variant functions of the rat and human enzymes.

The differential inhibition by AP sites and the identification of a specific protein-binding region in the N-terminal presequence of UNG1Δ29 may indicate that the 26 and 31 kDa UNG1 forms serve distinct functions. Both forms are, however, stimulated by HAP1, an AP endonuclease succeeding UNG proteins in the BER pathway. We have recently demonstrated that the rate-limiting step in catalysis by UNG1Δ84 is product release and that this may have a biological role in protecting cells from cytotoxic and mutagenic AP sites until further processed by HAP1 (25). It is thus tempting to speculate that the relative formation of 31 and 26 kDa forms of UNG1 may be regulated, perhaps to provide additional protection against the cytotoxic and mutagenic effects of unprocessed AP sites.

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