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Mitochondrial targeting of human DNA glycosylases for repair of oxidative DNA damage

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ABSTRACT

Oxidative damage to mitochondrial DNA has been implicated in human degenerative diseases and aging. Although removal of oxidative lesions from mitochondrial DNA occurs, the responsible DNA repair enzymes are poorly understood. By expressing the epitope-tagged proteins in COS-7 cells, we examined subcellular localizations of gene products of human DNA glycosylases: hOGG1, hMYH and hNTH1. A gene encoding for hOGG1 which excises 7,8-dihydro-8-oxoguanine (8-oxoG) from DNA generates four isoforms by alternative splicing (types 1a, 1b, 1c and 2). Three tagged isoforms (types 1b, 1c and 2) were localized in the mitochondria. Type 1a protein, which exclusively contains a putative nuclear localization signal, was sorted to the nucleus and lesser amount to the mitochondria. hMYH, a human homolog gene product of *Escherichia coli mutY* was mainly transported into the mitochondria. hNTH1 protein excising several pyrimidine lesions was transported into both the nucleus and mitochondria. In contrast to the three DNA glycosylases, translocation of the human major AP endonuclease (hAPE) into the mitochondria was hardly observed in COS-7 cells. These results suggest that the previously observed removal of oxidative base lesions in mitochondrial DNA is initiated by the above DNA glycosylases.

INTRODUCTION

A variety of DNA base modifications are generated by reactive oxygen species (ROS) and most of these lesions are excised by DNA glycosylases in living cells (1). *Escherichia coli*, the best characterized organism for the repair of oxidized DNA, uses different DNA glycosylases for purine and pyrimidine base lesions (2). A wide variety of oxidized pyrimidines are excised by *E.coli* endonuclease III. The general importance of this DNA glycosylase for life is suggested by the fact that many organisms whose complete genome sequences have been analyzed possess *E.coli nth*-like gene(s) and putative homologs are found in the database of various eukaryotic sequences from yeast to man (3). A gene product of the human homolog, termed hNTH1, was characterized as possessing a substrate specificity similar to that of *E.coli* endonuclease III (3,4). MutM/Fpg, another *E.coli* DNA glycosylase, acts on oxidized purines representing 7,8-dihydro-8-oxoguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (Fapy). The biological significance of the 8-oxoG is widely recognized because of its strong mutagenic potential and abundant production by ROS in cells. To counteract the 8-oxoG-induced mutations, *E.coli* provides two additional enzymes, namely, MutY that excises mispaired adenine opposite 8-oxoG, and MutT that acts as 8-oxo-dGTPase to sanitize the nucleotide pool. Human homologs of *mutY* and *mutT* have been cloned as *hMYH* (5) and *hMTH1* (6), suggesting that the repair of 8-oxoG is also crucial for human. Although a homolog of the *mutM* gene has not been identified in human, a gene encoding for a DNA glycosylase functionally similar to MutM, termed OGG1, was cloned initially from *Saccharomyces cerevisiae* (7,8) and recently from human as hMMH/hOGG1/hOGH1 (9–14). It is, therefore, reasonable to suppose that hOGG1, hMYH and hMTH1 cooperatively protect against the 8-oxoG-induced mutagenesis in human, as MutM, MutY and MutT in *E.coli* (15).

The apurinic/apyrimidinic (AP) site in DNA is generated by spontaneous base loss or by the action of DNA glycosylase. The AP site is toxic because it blocks DNA replication and is mutagenic when translesional DNA synthesis takes place (1). A major AP endonuclease, APE (APEX/HAP1/Ref-1), initiates the repair by nicking 5′-adjacent to the AP site. The incision leaves a 5′-terminal deoxyribose 5-phosphate (dRP) which disturbs the following repair synthesis. Experiments of *in vitro* reconstitution have shown that dRPase activity associated by DNA polymerase β (polβ) or nuclease activity of FEN-1 is required for the removal of the 5′-lesion and the subsequent repair synthesis (16,17). This base excision repair (BER) model appears to be limited to the APE-initiated repair and to the event in the nucleus. On the other hand, hNTH1 and hOGG1 are able to incise DNA strand at the glycosylitically produced AP site without APE due to the associated AP lyase activity. Thus, the process of repair initiated by the action of the DNA glycosylase/AP lyase remains to be established.

Mitochondrial oxidative energy metabolism is the major intracellular source of ROS. Mitochondrial biomolecules including mitochondrial DNA (mtDNA) are constantly exposed to a high

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extent of ROS. The accumulation of oxidative DNA lesions or the resulting mtDNA alteration has been implicated in the cause of aging and several human diseases such as Parkinson's disease and Alzheimer's disease (reviewed in 18–20). While the mitochondrial antioxidants such as superoxide dismutase, catalase and glutathione are well known to defend against ROS, the DNA repair mechanism and responsible enzymes in mitochondria are poorly understood. Nonetheless, observations that MutM- and endonuclease III-sensitive DNA lesions are efficiently removed from mtDNA (21,22), suggests the presence of DNA repair enzymes in this organella. Biochemical studies have shown that mitochondria contain endonuclease III-like AP nicking activity (23) and OGG1/MutM-like DNA glycosylase/AP lyase activity (24). Additionally, mitochondrial localization of hMTH1 has been documented, suggesting the existence of a repair and protection system against 8-oxoG-induced mutations (25). These studies prompted us to examine localization of products of DNA glycosylase genes recently isolated from human.

Among eukaryotic DNA glycosylases whose genes have been cloned so far, uracil DNA glycosylase (UNG) is the only protein for which mitochondrial localization has been demonstrated (26,27). It was revealed that two alternative forms are transcribed from the *UNG* gene, which have distinct N-terminal sequences: one containing a nuclear localization signal (NLS), and the other containing a mitochondrial targeting signal (MTS) (27). While single coding sequences for hMYH, hNTH1 and hAPE have been reported, we have previously identified four distinct hOGG1 transcripts generated by alternative splicing. All the isoforms (types 1a, 1b, 1c and 2) are equally able to reduce the mutation frequency of *E.coli* (*mutM mutY*) host cells (9). Here, we present the subcellular localization of the recombinant proteins of four hOGG1 isoforms, hMYH, hNTH1 and APE to assess the possibility that these gene products may be involved in the presumed mitochondrial base excision repair for oxidative damage.

MATERIALS AND METHODS

Plasmid constructs

Four hOGG1 cDNAs were cloned previously (9). The other cDNA encoding for hMYH, hNTH1 or hAPE was amplified by polymerase chain reaction (PCR) from human testis or brain cDNA library (Gibco-BRL) based on the database sequences (MYH, U63329; APE, D90373; NTH1, U81285). In order to tag the C-terminus of the protein, PCR was performed using a C-terminal primer containing a FLAG sequence and stop codon (GAC TAC AAG GAC GAC GAT GAC AAG TGA). The 5′-sequence was modified by introducing a translation signal (CCACC) in front of the initiation codon. The FLAG-tagged cDNA was cloned behind the CMV promoter of the expression vector, pTargeT (Promega). A fusion construct with green fluorescent protein (GFP) was obtained by subcloning a PCR product having appropriate restriction sites on the ends into pEGFP-C1 (Clontech).

Transfection

COS-7 cells, HeLa cells or CHO-9 cells grown in 35 mm tissue culture dishes were transfected with the above plasmid DNA using Lipofectamine (Gibco-BRL). The cells were washed in PBS and fixed with 4% paraformaldehyde in PBS at 44–48 h following the start of transfection. GFP fluorescence was then observed under a microscope. Transfectants with FLAG-tagged constructs were subsequently washed once, treated with 0.2% Triton X-100 in PBS for 2 min, washed twice, and incubated with a blocking reagent (NEN) in TN buffer (0.1 M Tris–HCl, 0.15 M NaCl, pH 7.5). The cells were then incubated with anti-FLAG M2 antibody (Kodak) at 40 µg/ml in TNT buffer (TN buffer containing 0.05% Tween 20) at room temperature for 1 h or at 4°C overnight. Indirect immunofluorescence was performed with a combination of a biotin-labeled second antibody (Zymed), a tyramide signal amplification system (TSA-Indirect, NEN) and a fluorescent conjugate (Streptavidin-Fluorescein, NEN) according to the manufacturer's instructions. For detection of mitochondria, MitoTracker Red CMXros (Molecular Probes) was added to the culture medium of the transfected cells 20 min before the fixation. The transfectants were visualized with a fluorescence microscope (Leica model DM LB) equipped with photographic system (MPS60). Filter L4 or TX (Leica) was used for the fluorescein and GFP or CMXros, respectively.

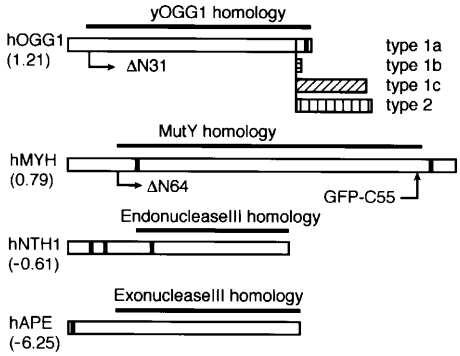

Figure 1. Schematic representation of possible NLS and MTS in human OGG1, MYH, NTH and APE proteins. PSORT (version II, http://psort.nibb.ac.jp/) was employed to predict nuclear and/or mitochondrial localizations. Scores of discriminant analysis for mitochondrial localization (MITDISC; 30) are shown in parentheses. Positions of the predicted NLSs are shown by filled boxes. The arrow represents a position used for construction of N-terminal truncated protein (ΔN31 or ΔN64) or that of GFP-fusion protein (GFP-C55).

RESULTS

Structure and MTS prediction of hOGG1, hMYH, hNTH1 and APE

A putative NLS has been suggested for hOGG1 (9–12,14), for hNTH1 (4) and for hAPE (29). We re-evaluated possible targeting signals for nucleus and mitochondria by using the PSORT program (30; Fig. 1). Four isoforms of hOGG1 (types 1a, 1b, 1c and 2) with a variable C-terminus of 8–108 residues are generated by alternative splicing (9). The putative NLS is exclusively found in the C-terminal segment of type 1a sequence. Human *MYH*, *NTH1* and *APE* are homologs of *E.coli mutY*, *nth* and *xth*, respectively. Their deduced sequences have 'extended' segments which are not included in the *E.coli* counterparts. The predicted NLSs reside mostly in the extended segments. For mitochondrial targeting, it is known that regular MTS is located at the N-terminal end composed of typically 20–60 residues with abundant positive charges, very little if any negative charges, and frequent hydroxylated residues which can form an amphipathic

Figure 2. Subcellular localization of hOGG1 isoforms in COS-7 cells. COS-7 cells were transfected with the constructs expressing C-terminal FLAG-tagged hOGG1 type 1a (**A**), type 1b (**B**) type 1c (**C**) and type 2 (**D**). The 31 amino acids of the N-terminal sequence are truncated from the type 1a construct (**E**) or from the type 2 constructs (**F**). Representative immunofluorescence staining with anti-FLAG M2 antibody (E) and (F) and left images in (A)–(D) and mitochondrial staining with CMXros [right images in (A)–(D)] are shown (magnification ×400).

α-helix (31). Mitochondrial targeting is predicted for hOGG1, hMYH and hNTH1.

Subcellular localization of hOGG1 isoforms

To access the actual localization of the hOGG1 isoforms *in vivo*, we transiently expressed a construct in COS-7 cells, in which a FLAG epitope was added at the C-terminus of the respective cDNA. The protein expression and its subcellular localization was detected by immunofluorescence staining using anti-FLAG M2 antibody. Cells were also treated with CMXros prior to fixation in order to visualize mitochondria.

As expected, the tagged type 1a protein was mainly detected in the nucleus. We observed, in addition to the nucleus, cytosolic fluorescence forming dots or lines characteristic of mitochondria (Fig. 2A). In sharp contrast to the nuclear translocation of the type 1a protein, the other three proteins were detected not in nuclei at all but in mitochondria (Fig. 2B–D). Therefore, the sequence PAKRRK at 333 in the C-terminal segment of type 1a is actually utilized for nuclear targeting and the common N-terminal domain contains a MTS. Similar localization for the each isoform was obtained in HeLa transfectants (not shown).

To confirm whether the common N-terminal region of hOGG1 protein possesses a regular MTS, a truncated protein with a deletion of N-terminal 31 amino acids, which retains the core domain homologous to *S.cerevisiae* OGG1, was expressed in COS-7 cells. The epitope-tagged ΔN31-type 1a protein was targeted to the nucleus without mitochondrial localization (Fig. 2E). The same N-terminal deletion from the type 2 protein (ΔN31-type 2) resulted in no mitochondrial localization, weak fluorescence from the nucleus and several bright granular dots in the cytosol (Fig. 2F), probably suggesting rapid transport and degradation in lysosome.

Subcellular localization of hMYH

In the bacterial repair and protection system against 8-oxoG-induced mutations, MutY and MutT also play substantial roles. However, in a *mutT*$^{-}$ background *E.coli* (*mutY*$^{+}$) is more mutagenic than *E.coli* (*mutY*$^{-}$) because MutY excises adenine opposite 8-oxoG that has been misincorporated by DNA

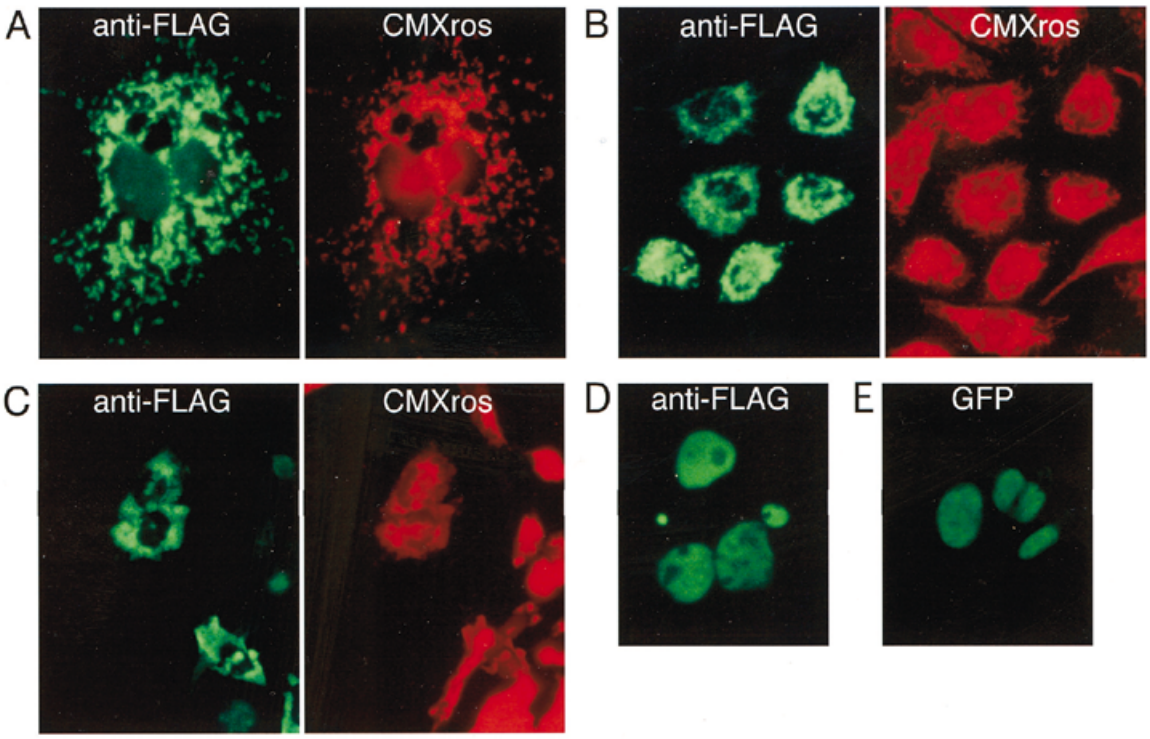

Figure 3. Mitochondrial and possible nuclear localization of hMYH protein. The hMYH-FLAG protein was transiently expressed in COS-7 cells (**A**), HeLa cells (**B**) or CHO-9 cells (**C**). The N-terminal truncated protein (ΔN64) or the GFP-fusion protein with the hMYH C-terminal segment (GFP-C55) was expressed in COS-7 cells [(**D**) or (**E**), respectively].

polymerase (32). In human cells, hMTH1 has been found in the mitochondrial matrix as well as in cytosol (25). This situation could allow hMYH protein to work in the mitochondria for cooperative DNA repair with hOGG1. Figure 4 shows the immunofluorescence staining of transiently expressed hMYH-FLAG protein. The protein was clearly co-localized with mitochondria in COS-7 cells (Fig. 3A). Unexpectedly, hMYH-FLAG protein was hardly detected in nucleus, although some transfectants which appeared to strongly express the recombinant protein showed both mitochondrial and nuclear localization of the protein (not shown). We further examined the localization of the hMYH-FLAG protein in different cell lines, HeLa cells (Fig. 3B) and CHO-9 cells (Fig. 3C). The results indicated mitochondrial localization of the protein and very little if any nuclear immunofluorescence in either type of cells.

According to the alignment (5), the human sequence, relative to *E.coli* MutY, has extended segments composed of 63 residues toward the N-terminus and ~50 residues toward the C-terminus. One NLS-like sequence (PWRRR at 98) is found in the core domain, but this is in the conserved block. A more likely NLS (RKKPR at 505) is located in the extended C-terminal segment (Fig. 1). To determine whether the latter C-terminal NLS is active or not, we expressed a GFP protein fused at its C-terminus with the C-terminal extended segment (55 residues) of the hMYH sequence (GFP-C55). The COS-7 transfectants showed nuclear localization of the protein (Fig. 3E). Furthermore, a construct in which the extended N-terminal segment (64 residues) was deleted from the hMYH-FLAG resulted in the nuclear localization of the ΔN64 protein (Fig. 3D). These results indicate that the C-terminal segment has a potential NLS.

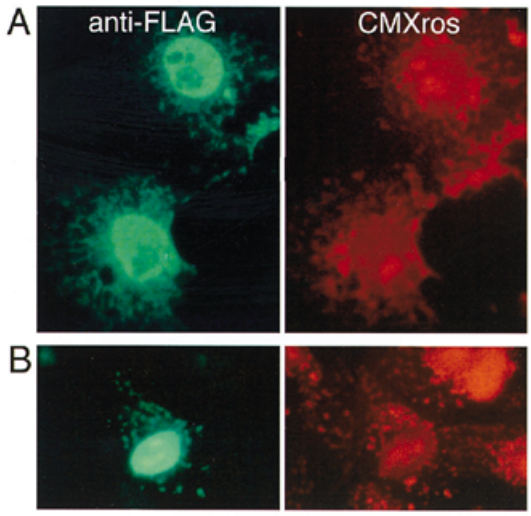

Figure 4. Dual localization of hNTH1 protein in nucleus and mitochondria. The hNTH1-FLAG protein was transiently expressed in COS-7 cells (**A**) or in HeLa cells (**B**) and visualized by immunofluorescence staining (left). Mitochondria were stained by CMXros (right).

Subcellular localization of hNTH1

We studied subcellular localization for another DNA glycosylase, hNTH1, to link the gene product to the observed mitochondrial repair activity for oxidative pyrimidines (21,22). The hNTH1 sequence has an extended N-terminal segment composed of ~95

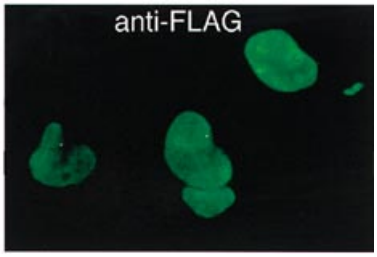

Figure 5. Nuclear localization of hAPE protein in COS-7 cells. The hAPE-FLAG protein was transiently expressed in COS-7 cells and visualized by immunofluorescence staining.

residues (3,4). N-terminal MTS and three putative NLSs appear within or near this segment (PLRRR at 30, PVKRPRK at 46, PKVRR at 117; Fig. 1). The expression of hNTH1-FLAG protein in COS-7 cells (Fig. 4A) and HeLa cells (Fig. 4B) resulted in the protein being transported into both the nucleus and mitochondria. Therefore, the hNTH1 protein has dual transport signals to repair nuclear and mitochondrial DNA lesions.

Subcellular localization of hAPE

In contrast to the above DNA glycosylases, N-terminal MTS is not predicted in the hAPE sequence while the NLS of hAPE is located near the end of the N-terminus (PKRGKK at 2). The extended N-terminal segment of mammalian APE is a domain, apart from the AP endonuclease activity, for the redox function to facilitate DNA binding of several transcription factors such as AP-1 (31). Although activity of hAPE protein is found mostly in nuclear extracts, immunohistochemical studies have shown differential nuclear and cytosolic localization of the protein depending on the tissues and cell types (34) and colorectal adenomas and carcinomas (35). We examined subcellular localization of hAPE-FLAG protein in COS-7 cells. As shown in Figure 5, the recombinant protein was predominantly localized in the nucleus. Mitochondrial and other cytosolic hAPE-FLAG protein was below the detection limit in the transfectants.

DISCUSSION

We used an epitope-tagging strategy to demonstrate the subcellular localization of hOGG1, hMYH, hNTH1 and hAPE proteins. Results of the nuclear localizations of hOGG1 type 1a and hNTH1, together with recent evidence that the nucleotide excision repair (NER) removes thymine glycol and 8-oxoG *in vitro* (36), indicate that the nucleus has two distinct pathways for repair of the same lesions. More importantly, the mitochondrial localizations of four hOGG1 isoforms and hNTH1 imply that these DNA glycosylases are the requisite enzymes for the repair of mtDNA. Although the specific contribution of hOGG1 and hNTH1 to the repair is uncertain, the substrates characterized to date for hOGG1 (8-oxoG and Fapy; 9,11–14), and hNTH1 (thymine glycol and urea; 3,4) are the most abundant DNA lesions generated by ROS and are included in the MutM- and endonuclease III-sensitive lesions, efficient removal of which from mtDNA has been observed previously (21,22,37). Since mitochondria do not appear to possess NER for the removal of these lesions, our results underscore the importance of mitochondrial BER for these oxidative lesions initiated by hOGG1 and hNTH1 DNA glycosylases. In addition, we showed that hMYH is also targeted to mitochondria. Together with the previously demonstrated mitochondrial localization of hMTH1 (25), hOGG1, hMYH and hMTH1 can constitute mitochondrial repair and protection system against the 8-oxoG-induced mutagenesis.

The molecular weight of the hOGG1 isoform varies with the length of the each C-terminal segment. Type 1a (39 kDa) and type 1b (36 kDa) are small, consisting of a core domain conserved between yeast and human, while type 1c (46 kDa) and type 2 (47 kDa) have a relatively long C-terminal segment with sequences unrelated to each other (Fig. 1). An 8-oxoG DNA glycosylase/AP lyase purified from rat liver mitochondria has a molecular mass of 25–30 kDa as estimated by gel-filtration (38). When considering that a presequence (usually 20–60 residues) is processed in the mitochondrial matrix, the purified protein could be a mitochondrial form of a rat OGG1 protein, presumably corresponding to the small human isoform (type 1a and/or type 1b).

hOGG1 splicing variants were obtained from human colon cDNA (9). These transcripts seem to be ubiquitously expressed when analyzed by RT–PCR and at least type 1a and type 1b are expressed at similar level by RNase protection assay (9; H.Aburatani and Y.Hippo, unpublished). Also, partial sequences for all the isoforms from various sources have been entered in the EST database. It is an interesting question why human cells express four isoforms all directed to mitochondria. Recently, a limited substrate specificity of *S.cerevisiae* OGG1 (a counterpart of human type 1a) has been reported, where the enzyme does not excise several purine lesions including adenine lesions 8-hydroxyadenine and 4,6-diamino-5-formamidopyrimidine (39). The variable C-terminal domain in type 1c and type 2 might provide different or additional substrate specificity in order to manage the repair for such minor purine lesions in mitochondria in the absence of NER.

The *hMYH* gene encoding for a 59 kDa protein with a 41% amino acid sequence similarity to *E.coli* MutY was cloned from a database search with the bacterial sequence (5). While the activity of the gene product has not been published, a 65 kDa protein with the adenine DNA glycosylase activity and the cross-reactivity to anti-MutY antibodies has been purified from calf thymus and HeLa cells, which is also termed MYH (40). We presumed that the *hMYH* gene encodes for the latter purified protein and used the nucleotide sequence of the gene to amplify the cDNA. We thus initially expected nuclear localization of the recombinant protein because the protein had been obtained from the nuclear extracts (40). However, translocation of the full-length hMYH-FLAG protein into nuclei was relatively weak in COS-7 cells and almost undetectable in HeLa and CHO-9 cells. The results of the GFP-fusion and the N-terminal truncated protein indicate that the C-terminal sequence of hMYH contains a potential NLS. Therefore, we conclude that the NLS of hMYH protein is less effective than the N-terminal MTS as a protein transport signal. However, a discrepancy remains between calf thymus nuclear extracts containing MYH protein activity and the apparent absence of the recombinant hMYH protein in HeLa/CHO-9 transfectants (Fig. 3B and C). It is possible to consider the existence of a second, yet similar, hMYH protein in the nucleus. With regard to this, the exclusive localization of UNG protein has been found to be originated by alternative splicing (28). The second hMYH, if present, might be an isoform derived from an alternative splicing of the *hMYH* gene where the N-terminal MTS

is deleted or replaced. Further work is needed to identify the gene providing the nuclear MYH activity.

A BER repair model for AP sites has been proposed, which includes APE incision and polβ- or PCNA-dependent processing (16,17). Although the model is not fully applicable to mitochondrial BER, incision at AP sites is expected to initiate the repair. Recently, a class II mitochondrial AP endonuclease of *Xenopus laevis* has been revealed to initiate the AP-DNA repair *in vitro* (41). Our result showing apparent nuclear localization of hAPE suggests that the mitochondrial AP endonuclease might be an alternative form of *Xenopus* APE or encoded by a distinct gene. On the other hand, an early study showed other AP nicking activities in mouse plasmacytoma mitochondria (23). The enzymes are EDTA resistant and incise UV-irradiated DNA in addition to AP-DNA. These properties and the mitochondrial localization of hNTH1 shown here suggest that at least one of the mitochondrial AP nicking activities is an AP lyase function of NTH1 protein. The contribution of the mitochondrial AP lyase to the AP-DNA repair and the processing of 3′-unsaturated sugar–phosphate generated by it remain to be established.

MtDNA alterations including deletion, rearrangement, point mutation and fragmentation are age-associated events (18–20). Oxidative damage is thought to be a primary cause of these mutations which lead to mitochondrial dysfunction and then increased ROS production (20). Normal mitochondrial function seems to be required for maintenance of the mitochondrial DNA repair capacity because oxidative lesions in mtDNA are accumulated in aged cells, and are unrepaired in cells under strong oxidative stress which does not affect the nuclear DNA repair capacity (42). Elucidation and characterization of the mitochondrial DNA repair enzymes involved in BER and also mismatch repair is an important step toward understanding the molecular basis of age-associated mitochondrial genome instability.

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