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Biochemical Characterization of Human AlkBH7

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By

Matthew Lawrence Bobiak

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Abstract of the Dissertation

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The human mitochondrial genome (mtDNA) is essential for normal cellular function. Encoded within the 16.6 kilobase circular genome are 13 proteins, 22 tRNAs and 2 rRNAs which allow for efficient cellular ATP production. The 13 proteins encoded by the mtDNA represent an estimated 1% of the total proteins present in the mitochondria as the vast majority of proteins are nuclear gene products imported into mitochondria. In order to maintain normal cellular respiration, replication and repair of the mitochondrial genome is carried out by proteins encoded in the nucleus and imported into the mitochondria. Like the nuclear genome, the mtDNA is subject to endogenous and exogenous stress that can result in DNA damage which, if unrepaired, can cause mutation, deletion and depletion of the mtDNA. In nuclear DNA, genomic insults are mitigated by a series of processes including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and direct repair. NER has not been demonstrated in mitochondrial extracts, the activity of MMR in mammalian mitochondria

is controversial, but unlikely, and few DNA glycosylases enter mitochondrial to participate in BER. The contribution of direct repair to maintenance of mtDNA has not been characterized.

The E. coli AlkB protein is a Fe^{2+}- alpha-ketoglutarate (αKG) dependent dioxygenase. Long associated with the adaptive response to DNA damage, the biochemical activity of AlkB remained elusive until bioinformatic analysis suggested Fe^{2+} and αKG as essential cofactors. The reactions catalyzed by AlkB indicate that it functions as a direct repair enzyme capable of removing exocyclic adducts from DNA via a direct removal mechanism, similar to methyl-guanine methyl transferase. Bioinformatic analysis suggested that AlkB has at least 9 homologs in the human genome. Analysis of the 9 human AlkB homologs suggests that AlkBH7 has a high probability for mitochondrial targeting and may contribute to repair of the mtDNA. Predictions of localization of AlkBH7 to the mitochondria have been confirmed using microscopic and biophysical separation techniques. *In vitro* biochemical analysis confirmed the ability of recombinant AlkBH7 to catalyze direct reversal of 3-methylcytosine and 1-N^6 ethenoadenine. Confirming the prediction that AlkBH7 can contribute to repair of the mitochondrial genome *in vivo*, expression of AlkBH7 confers modest resistance to chloroacetaldehyde on both the cellular and molecular level. This work represents the first description of the biochemical properties of AlkBH7 and the first demonstration that direct repair contributes to the maintenance of human mtDNA.

Dedication

This dissertation is dedicated to my grandparents Charles and Veronica Zapiec and Adolph and Elizabeth Bobiak. Without their support and counsel throughout life, this dissertation would not have been possible. They have provided me with models of hard work, dedication and devotion that have contributed to my academic and personal development. My parents Lawrence and Joanne Bobiak, deserve immense credit and utmost thanks for their love and support; this dissertation would not have be possible without them. Their commitment to education has encouraged me throughout my academic career.

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I have been extremely fortunate to have many great role models in life. My youth experience with the Boy Scouts of America has provided me exposure to many great men after whom I have attempted to model myself. William Thomas, Egbert Horton and Jim Davis were models of practical wisdom, rational problem solving, ethical leadership, kindness and generosity. Incorporating these qualities into my daily life has thoroughly enriched my life and my interactions with others.

Special thanks are owed to the employees and volunteers at the Hamlet Organic Garden, specifically Sean and Jill Pilger. My involvement with the farm provided the

antithesis to the grind of graduate school, and made significant contribution to my physical, mental and emotional wellbeing. I will always be grateful for the friendship and support offered by the HOG community and cannot express the degree to which they have contributed to this dissertation.

Contents

List of Abbreviations

1-meA	1-Methyladenine
3-meC	3-Methylcytosine
α-KG	Alpha-Ketoglutarate
AA	Amino Acid(s)
AAG	Alkyladenine Glycosylase
ADP	Adenosine Diphosphate
APE	Apurinic/ Apyrimidinic Endonuclease
ATP	Adenosine Triphosphate
BER	Base Excision Repair
CAA	Chloroacetaldehyde
dNTP	Deoxynucleotide Triphosphate
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
εA	1-N^6 Ethenoadenine
εC	3-N^4 Ethenocytosine
EDTA	Ethylenediaminetetracetic Acid
$FADH_2$	Flavin Adenine Dinucleotide (reduced)
GSA	Glycosylase Sensitivity Assay
LPO	Lipid Peroxidation
M1G	Pyrimido[1,2-*a*]purin-10(*3H*)-one
MMS	Methyl methanesulfonate

mtDNA	Mitochondrial DNA
MTS	Mitochondrial Targeting Sequence
NADH+	Nicotinamide Adenine Dinucleotide (reduced)
pBS (+/-)	pBlueScript
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl Fluoride
RFP	Red Fluorescent Protein
ROS	Reactive Oxygen Species
ssDNA	Single-stranded DNA
TX-100	Triton X-100

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This dissertation would not have been possible without my advisor Dr. Daniel Bogenhagen. He has instilled in me a commitment to rigorous, rational, scientific thought, and professional ethics which I hope to perpetuate throughout my career. My dissertation committee, Dr. Orlando Schärer, Dr. Masaaki Moriya and Dr. Paul Fisher deserve special thanks for their involvement in my intellectual development.

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Chapter 1: General Introduction

Mitochondrial Biology

The 16.6 kB human mitochondrial genome (mtDNA) encodes 13 proteins, 22 tRNAs and 2 rRNAs that are required for proper metabolic function (Anderson et al., 1981). The encoded proteins are subunits of electron transport chain complexes I, III and IV, and ATP synthetase. The tRNA and rRNA molecules allow for translation of these 13 proteins within the mitochondria. The mitochondrial proteome is much more complex than the mitochondrial genome suggests. Not only are mitochondria centers for cellular energy production, they also are responsible for heme biosynthesis (Ajioka et al., 2006), steroid synthesis (Christenson, 2000), and calcium storage (Nicholls, 2004). Mitochondria are intimately involved in regulation of cellular homeostasis and control of apoptosis. These mitochondrial functions, in addition to replication and transcription of the mitochondrial genome, depend on proteins encoded in the nucleus which are imported to the mitochondria. Studies of the mitochondrial proteome suggest that between 900 and 1,500 proteins are present in the mitochondria of human cells (Da Cruz et al., 2003; Forner et al., 2006; Gaucher et al., 2004; Hopper et al., 2006). A subset of these proteins associates with the mtDNA creating an ultrastructural organization center termed the mitochondrial nucleoid (Bogenhagen et al., 2003; Kienhofer et al., 2009; Satoh, 1991; Wang, 2006). Nucleoid proteins have canonical roles in replication, transcription, translation, and repair of the mtDNA as well as with metabolic processes, nucleotide import/ export, and microtubule association (Bogenhagen et al., 2008).

Mitochondria are double lipid membrane bound subcellular organelles. The outer membrane is highly porous while the inner membrane is extensively folded and rather impervious to charged particles. Mitochondria exist as a dynamic intracellular network which undergoes fission and fusion (Detmer, 2007; Olichon et al., 2006; Suen et al., 2008) while intracellular movement of mitochondria is guided by microtubule association (Boldogh et al., 2003). Mitochondrial nucleoids are contained in the mitochondrial matrix, bound by the inner mitochondrial membrane, but are associated with the inner mitochondrial membrane. Import of nuclear- encoded mitochondrial matrix proteins requires the assistance of TOM (Transporter Outer Membrane) and TIM (Transporter Inner Membrane) complexes.

The most critical function of the mitochondria is cellular respiration via electron transport and oxidative phosphorylation. Pyruvate is imported to the mitochondria where it undergoes oxidative decarboxylation by pyruvate dehydrogenase to produce acetyl-CoA and $NADH^+$ and releasing CO_2. Acetyl-CoA enters the tricarboxcylic acid (TCA) cycle producing $NADH^+$ and $FADH_2$ which provide electrons to complex I and II, respectively. During electron transport through complexes I, III and IV, protons are pumped into the intermembrane space of the mitochondria generating a pH gradient of about 0.5 pH units. Re-entry of protons into the matrix through the rotary motor of ATP synthetase drives phosphorylation of ADP + PO_4 to ATP. The proteins encoded by the mtDNA are components of the electron transfer and ATP synthetase complexes making the integrity of the mitochondrial genome essential for efficient energy production.

Diseases Associated with mtDNA mutation

Mitochondrial diseases can be divided into two broad classes resulting from either nuclear gene defects or mitochondrial DNA mutations. Aberrations in the mitochondrial genome in the form of point mutations, deletions, DNA depletion result in a vast array of disorders.

Point mutations in the mtDNA have been linked to aminoglycoside treatment dependent neurosensory hearing loss (DEAF) (Estivill et al., 1998), Leber's Hereditary Optic Neuropathy (LHON), Myoclonic Epilepsy with Ragged Red Fibers (MERRF), Mitochondrial Myopathy with Encephalopathy, Lactic Acidosis, and Stroke (MELAS), Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa (NARP) (Kogelnik et al., 1998) (Figure 1.1). Because the genetics of mtDNA inheritance and segregation are more complex than simple Mendelian genetics would suggest, it is often difficult to ascertain that mtDNA mutation is the cause of a disease.

Mitochondrial deletions have a significant role in mtDNA-associated disease. Many deletions ranging in size from 5 to 10,000 bases have been described. Often these mutations occur between direct repeats of the mtDNA. Pathologies associated with mtDNA deletions include encephalomyopathies, muscle weakness, fatigue, ataxia, and maternally inherited diabetes. Kearns-Sayer Syndrome (KSS) results from a 5 kB deletion of the mtDNA shown in Figure 1.1. Often these deletions exist in a heteroplasmic state with normal mtDNA molecules. The incidence of deletions increases with age in part because the deleted genomes that retain origins of replication may replicate more rapidly than normal mtDNA molecules.

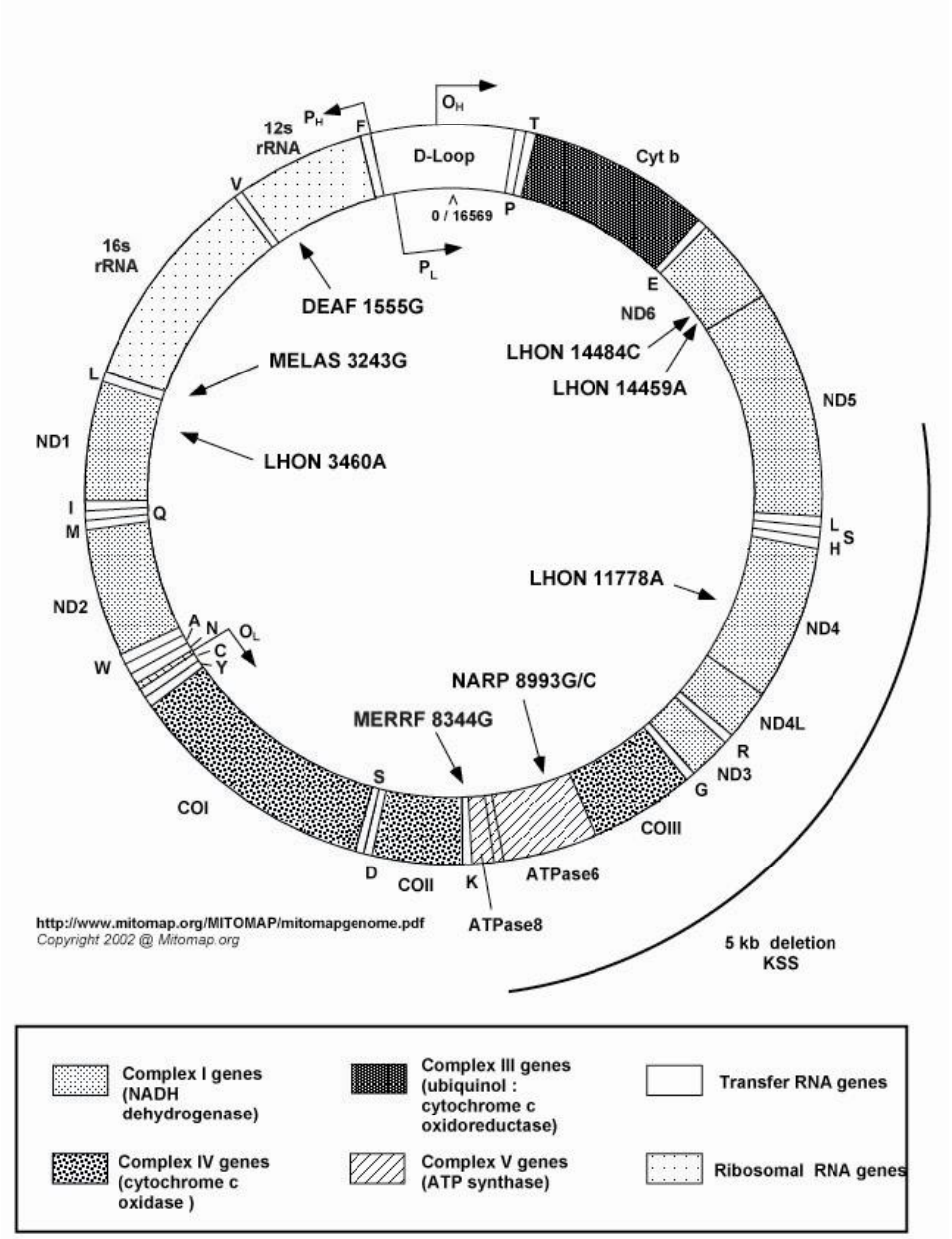


Figure 1.1 Organization of the Human Mitochondrial Genome. Significant features of the mtDNA shown include the control region (D-Loop), 13 protein encoding genes, 22 tRNA genes and 2 rRNA genes. Also shown are common disease-associated mutations. Image taken from MITOMAP: A Human Mitochondrial Genome Database. http://www.mitomap.org, 2009.

Mitochondrial depletion can result from genetic disorders or pharmacologic inhibition of mtDNA replication, depletion of essential replication substrates or excessive damage to the mtDNA. Deficiency of mitochondrial thymidine kinase or deoxyguanosine kinase can lead to tissue-specific mtDNA depletion (Bornstein et al., 2008; Hirano et al., 1994; Salviati et al., 2002) (Nishigaki et al., 2003). The abundance of nucleotides required to replicate the mtDNA can affect mtDNA abundance (Ferraro et al., 2006; Ferraro et al., 2005). Temporary mitochondrial depletion can result from folate deficiency, necessary to generate deoxythymidine (Crott et al., 2005; James et al., 1994). Nucleoside analog inhibitors such as azidothymidine (AZT) used to treat human immunodeficiency virus (HIV) infection, can inhibit pol γ replication (Lim, 2001). Excessive alcohol consumption results in significant transient mtDNA depletion in the liver (Bailey, 1998; Cahill et al., 1999; Cahill et al., 1997; Coleman, 1990; Mansouri et al., 1997; Mansouri et al., 1999).

While rare, severe clinical diseases can result from mitochondrial mutation, deletion or depletion, two common processes, aging and neurodegeneration are associated with impaired mtDNA replication. Mice expressing an exonuclease deficient pol γ show a premature aging phenotype and massive mutation of the mtDNA (Trifunovic et al., 2004; Kujoth et al., 2007). The links between parkinsonism and Alzheimer's disease and mtDNA have not yet been definitely proven, however a strong association can be made between these factors (Coskun et al., 2004; Floyd, 2002; Kish et al., 1999; Ojaimi et al., 1999).

Replication of the Mitochondrial Genome

Efficient replication of the mitochondrial genome is carried about by DNA polymerase gamma (pol γ). The catalytic subunit of pol γ (pol γA) associates the accessory subunit pol γB in a heterotrimeric fashion (γAB_2) forming a highly processive enzymatic complex (Carrodeguas et al., 1999; Yakubovskaya et al., 2006). Association of the γAB_2 complex with the T7-like (twinkle) helicase forms the minimal replicative complex in vitro (Farge et al., 2007; Tyynismaa et al., 2004).

In contrast to symmetrical bidirectional DNA replication observed during nuclear DNA replication, mitochondrial DNA replication occurs asymmetrically (Clayton, 1991; Clayton, 1992; Doda et al., 1981). Due to unequal distribution of purines and pyrimidines on the complementary strands of the mtDNA, the strands can be differentiated by the monikers "heavy" and "light." Both the transcription and replication processes originate in a region denoted the mitochondrial D-Loop. Within the D-Loop, mtDNA replication is initiated at an origin of replication in the heavy strand (O_H) and proceeds asymmetrically over roughly 2/3 of the mitochondrial genome until the light strand origin of replication (O_L) is encountered. The non-replicated light strand is displaced and protected by association with mtSSB (Pavco, 1985; Takamatsu et al., 2002; VanTuyle, 1985). Initiation events with the D-Loop have been suggested to be bidirectional (Holt et al., 2000) but only asymmetric extension from the O_H generates productive replication (Bogenhagen et al., 1979; Fish et al., 2004). Once the O_L is encountered, replication then proceeds along both strands to generate mature double-stranded replicates of the mtDNA (Brown et al., 2005).

Replication of the mtDNA is controlled by several mechanisms. DNA replication is primed by transcription from the light strand transcription start site, thus associating transcription with replication (Lee, 1998). Modulated expression of mitochondrial transcription factors such as TFAM and mTERF can modulate mtDNA levels (Hyvarinen et al., 2007; Larsson et al., 1994). Mitochondria are highly dynamic and move throughout the cytoplasm of a cell; however replication seems to occur in a perinuclear region, thus implying an association of the intracellular location with mtDNA replication (Davis, 1996). Regulation of nucleotide pools can provide further regulation. MtDNA replication is cell cycle independent requiring a supply of dNTPs in G_1 and G_2 as well as during S phase (Bogenhagen, 1977). Mitochondrial isoforms of thymidine kinase (TK2) and deoxyguanosine kinase (DGUOK) maintain sufficient supplies of dNTPs to support mtDNA replication. Additionally, evidence suggests that RRM2B, a p53 dependent protein that regulates ribonucleotide reduction helps maintain a suitable dNTP pool for mtDNA replication (Bornstein et al., 2008; Bourdon et al., 2007; Kollberg et al., 2009).

Repair of the Mitochondrial Genome

As with nuclear DNA, the mitochondrial genome is subject to replication errors as well as spontaneous mutagenesis by endogenous and exogenous factors. Misinsertions by pol γ can be eliminated by the exonuclease activity of the native enzyme, thus increasing replicative fidelity (Insdorf, 1989; Longley et al., 2001). However, this process is not error-free, and those mismatches that escape this immediate editing may persist in mtDNA due to the apparent absence of an effective mismatch repair process.

The mitochondrial genome is a significant target for oxidative DNA damage. While the nuclear DNA is protected by a full complement of DNA repair processes,

including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination, non-homologous end joining (NHEJ), and direct reversal, the mitochondrial genome has only been demonstrated to be repaired by BER.

BER utilizes lesion-specific glycosylases to detect and excise modified bases from the DNA, yet only a small number of glycosylases have been shown to be present in the mitochondria. 8-oxo-guanine glycosylase (OGG1) and uracil DNA glycosylase recognize and excise 8-oxoguanine and uracil respectively and have been shown to be mitochondrial (Croteau et al., 1997; Nishioka et al., 1999; Slupphaug et al., 1993). A mitochondrial isoform of NthI, an endonuclease III homolog, recognizes and excises thymine glycol mismatches from the mtDNA (Ikeda et al., 2002). A mitochondrial MutY homolog recognizes and excises the incorrect base from A/G or A/C mis-insertion events as well as removing an undamaged A or G opposite an 8-oxoG base (Ohtsubo et al., 2000). Mitochondrial DNA glycosylases that can contribute to repair of methylated or LPO-modified bases, activities attributed in the nuclear genome to alkyladenine DNA glycosylase (AAG) or mismatch specific thymidine DNA glycosylase (TDG), have been notably absent from description of mitochondrial DNA repair activities.

Generation and Mitigation of Reactive Oxygen Species (ROS) by the Mitochondria

As the terminal electron acceptor for the electron transport chain, O_2 plays an essential role in energy production. Between 98 and 99% of O_2 is fully oxidized to H_2O during the process; however, reactive oxygen species (ROS), oxygen-containing compounds with unpaired outer shell electrons (free radicals) or highly unstable

peroxides and superoxides can be generated during the process (Stryer et al., 2002). ROS can react with nucleic acids, proteins or lipids through free radical chemistry. To protect against the activity of superoxides, a cellular defense has evolved. Superoxide dismutase (SOD) enzymes catalyze the dismutation of $^{-}O_2$ to hydrogen peroxide (H_2O_2). H_2O_2 is decomposed by catalase or glutathione peroxidase to H_2O and O_2, thereby detoxifying most of the ROS produced by electron spillage. Some ROS molecules can however escape containment and initiate oxidation or free radical reactions.

ROS damage to the genetic material is of particular concern as the resulting base modification and phosphodiester backbone breakage can result in heritable mutations. A set of DNA glycosylases, which can recognize and initiate the repair of oxidatively modified bases, have evolved as initiators of BER (David et al., 2007; Gros et al., 2003). Abasic sites generated by spontaneous base loss enter the BER cycle through the action of AP endonucleases (Barzilay, 1995; Masuda et al., 1998). Reaction of ROS with lipid membranes can cause lipid peroxidation (LPO) yielding reactive lipid aldehydes (Marnett, 2002). These reactive aldehydes can target the same biomolecules as the parent ROS causing secondary ROS adducts. Primary LPO products can generate DNA base modifications (Blair, 2008; Ghissassi et al., 1995). These lesions can be processed by either by DNA glycosylases such as alkyladenine glycosylase (AAG) and the mismatch specific thymidine DNA glycosylase (TDG) or homologs of the E. coli AlkB protein (Delaney et al., 2005; Gros et al., 2003).

Given the proximity of the mitochondrial genome to the ROS generated by the electron transport chain, and the association with the inner mitochondrial membrane, mtDNA represents a significant target for primary ROS action and secondary ROS

effectors. MtDNA is more sensitive to H_2O_2 than nuclear DNA (Yakes, 1997). M1G, a marker for LPO, is readily detectible in mtDNA (Jeong et al., 2005). 8-oxoguanine, the most abundant oxidative damage to DNA, is 10-20 fold more abundant in mtDNA than nuclear DNA. To protect the mtDNA from 8-oxo-G, the 8-oxo-guanine glycosylase (OGG) and a MUTY homolog (MYH) which excises adenine bases inserted opposite 8-oxo-G during replication have isoforms that are found in the mitochondria.

Lipid Peroxidation and DNA Damage

ROS react in non-specific fashions. As general oxidants, they can interact with proteins, carbohydrates, lipids and nucleic acids that constitute the biological molecules of a cell. While potentially biologically significant, the roles and consequences of ROS-modified carbohydrates are beyond the scope of this discussion. Protein modification by ROS can serve biological functions as in the case of PTP1b (Tonks, 2005) or can generate protein modifications that cause impaired function or degradation. For example, tyrosine nitrosylation and oxidation of Fe-S complexes have been documented in mitochondrial proteins (Murray et al., 2003). ROS can act on polyunsaturated fatty acids to initiate degradation into bifunctional aldehydes (Blair, 2008). These bifunctional aldehydes can further react with GSH, proteins and nucleic acids. As the spectrum of bifunctional aldehydes formed is broad, so are the lesions they generate. 4-oxo-2(E)-nonenal (ONE) and 4-hydroperoxy2(E)-nonenal (HPNE) can interact with DNA to create unsubstituted and substituted etheno-DNA adducts respectively. Malondialdehyde forms a cyclic propano-DNA adduct with guanine (M1G) (reviewed in (Pluskota-Karwatka, 2008).

Etheno-adducts cause replication stalling and mutagenesis (Levine et al., 2000; Pandya, 1996; Tolentino et al., 2008). The most common etheno-DNA adduct occurs on the N^1 position of adenine resulting in the 1-N^6 ethenoadenine (εA). Such lesions can be generated by exogenous DNA damaging agents as well as by endogenous LPO. Vinyl carbamate, a product of vinyl chloride metabolism, can form etheno-adducts (Green, 1978). Similarly, etheno-adducts can result from exposure to chloroacetaldehyde in vitro and in vivo. The inability of replicative polymerases to create a stable base pair with εA causes stalling of the replication fork. As such, the necessity to remove εA from DNA to mitigate mutagenesis is critical to maintain fidelity of the genomic information.

Repair of LPO lesions has been attributed to multiple processes. Unsubstituted etheno-adducts can be excised by AAG (εA) and hsTDG (3N^4 ethenocytosine, εC) utilizing BER (Gros et al., 2003; Lee et al., 2009; Saparbaev, 1998). M1G is thought to be eliminated by NER (Knutson et al., 2007), a pathway unavailable in the mitochondria. Little is known regarding the elimination of substituted etheno-adducts. They can be bypassed by X family polymerases in vitro and by an uncharacterized enzyme in vivo (Pollack et al., 2006; Yang et al., 2009b). Bypass polymerases can contribute to overcoming replication stalling due to unsubstituted etheno-DNA lesions. Human Y family polymerases eta and kappa allow for polymerase progression when εA is present in the template strand by either an error-prone insertion or frame shifting deletion mechanism (Levine et al., 2001). X family human polymerase mu can bypass εA by inserting C or G nucleotides paired adjacent to the adducted strand (Zhang et al., 2002). However, these enzymes are not known to function in mitochondria. Thus, LPO damage to mtDNA may be relatively refractory to repair. Much of the research in this dissertation

was stimulated by the finding that the E. coli protein AlkB can catalyze the direct removal of the unsubstituted etheno-adducts from DNA (Delaney et al., 2005).

DNA Repair by AlkB

The E. coli protein AlkB has been associated with the adaptive response (ADA) to DNA damage by methylating agents (Volkert, 1988). ADA encodes a set of proteins that allows increased viability following exposure to methylmethane sulfonate (MMS) and methylnitronitrosoguanidine (MNNG). The proteins associated with ADA activation include AlkA , also known as the E. coli alkyladenine glycosylase, Ada protein, the E. coli homolog of the methylguanine methyltransferase (MGMT), AlkB and aidB.

As a DNA glycosylase, the activity of AlkA is well characterized. AlkA contributes to BER by recognizing and excising a subset of methylated purines including 3-methyladenine. While distinct on the sequence level, AlkA is the functional equivalent of methyl-purine glycosylase (MPG, aka ANPG) and AAG. AlkA/ MPG/ MNPG/AAG catalyze the removal of a broad set of modified base substrates including 3-methyladenine and hypoxanthine (Lee et al., 2009). Ada/MGMT protein is a "suicide enzyme" which transfers aberrant methylations from the O^6 position of guanine to itself thereby inactivating the enzyme (Lindahl, 1982). Additionally, Ada protein acts as a transcription factor which regulates expression of adaptive response genes (Saget, 1994).

Deletion of AlkB in vivo results in increased sensitivity to MMS (Kataoka et al., 1983). The catalytic activity of AlkB remained elusive until bioinformatic analysis suggested that the amino acid sequence contained domains conserved in Fe^{2+}, alpha-ketoglutarate dependent dioxygenase (Aravind, 2001). Utilizing these conditions it has

been shown that AlkB catalyzes DNA repair or 1-methyladenine, 3-methycytosine, 5-methylguanine releasing the methyl groups as formaldehyde (Falnes et al., 2002; Trewick et al., 2002). In vitro substrates include both DNA and RNA but a preference for ssDNA is exhibited. Additionally, the ability of AlkB to catalyze the direct reversal of ethano- and etheno- bridges from ethanoadenine and ethenoadenine has been demonstrated with the ethano-/ etheno- moiety being released as glyoxal (Delaney et al., 2005; Frick et al., 2007; Young Kim et al., 2007).

AlkB has 8 direct homologs in the human genome (Kurowski et al., 2003). Additionally, a protein known as FTO contains an AlkB domain and has retained minimal catalytic activity, although the association of this activity with the obesity-related phenotype of FTO knockout mice remains uncertain (Gerken et al., 2007). Each of the AlkB family members contains an active site core comprised of HXD and RXXXXXR motifs which allow for Fe^{2+} and α-KG binding (Aravind, 2001). Additionally, catalytic activity of the enzyme family depends on an oxidized leucine in the active site to interact and orient α-KG (Sundheim et al., 2006). Prior to this work, catalytic competency of AlkB homologs 1, 2, 3, 4, 6 and FTO have been investigated using a subset of substrates including 1-meA, 3-meC, 1-methylguanine, 3-methylthymidine, ethanoadenine and εA (Delaney, 2004; Delaney et al., 2005; Duncan et al., 2002; Gerken et al., 2007; Lee et al., 2005). Human AlkB homologs 1, 2, 3 and FTO have been shown to be capable of catalyzing the removal of the offending DNA adduct resulting in the restoration of the native nucleobase.

Crystal structures of AlkB, AlkBH2 and AlkBH3 have been ascertained. The catalytic core of AlkB proteins consists of 8 β-sheets forming a jellyroll structure (Yu et

al., 2006). Two α-helices encoded at the amino-terminal end of the protein provide structural rigidity but have little influence on the catalytic function. Structures determined in conjunction with modified DNA provide insight into the mechanism of DNA binding and base flipping by AlkB proteins. AlkB compresses the lesion-containing strand, creating significant distortion of the DNA resulting in the adducted base being flipped into the active site. AlkBH2 and 3 contain extended loops that stabilize the protein-DNA association. The adducted base is flipped into the active site by the insertion of a phenylalanine finger that stacks with the neighboring bases maintaining the overall structure of the DNA helix (Sundheim et al., 2008).

Hypothesis

This dissertation describes the research undertaken to investigate the contribution of direct repair by human AlkB homologs on the maintenance of the mitochondrial genome. It was reasoned that LPO lesions such as εA and εC may be generated in mtDNA and may not be repaired by BER due to the lack of DNA glycosylases responsible for initiating this process in the mitochondria. I developed the hypothesis that direct removal of these lesions by AlkB family members may contribute to the overall maintenance of the mtDNA. To address this question, bioinformatic analysis of the human AlkB homologs was employed to select AlkBH7 as a candidate mitochondrial DNA repair protein. Subcellular localization studies employing both microscopic and physical separation techniques were undertaken. The biochemical activity of recombinant protein was studied. Finally, techniques to assess cellular viability and mtDNA integrity in response to chloroacetaldehyde were used to demonstrate the ability

of AlkBH7 to contribute to repair of the mitochondrial genome in response to etheno-lesions.

Chapter 2: Analysis of Human AlkB Homologs for Mitochondrial Targeting and Study of the Predicted Secondary Structural Elements of AlkBH7

Introduction

Eight direct human homologues of AlkB have been described (Kurowski et al., 2003). Structural analysis of AlkB, AlkBH2 and AlkBH3 suggest that the protein family adopts a β jellyroll structure which supports the catalytic activity as direct removal enzymes (Ringvoll et al., 2008; Sundheim et al., 2008; Yang et al., 2008; Yu et al., 2006). As the mtDNA is located within the mitochondrial matrix, any AlkB family proteins that contribute to repair of the mtDNA should have the potential to adopt similar folds and mitochondrial targeting elements which would allow the protein access to the mitochondrial matrix.

Mitochondrial proteins are sorted by four distinct mechanisms (Bolender et al., 2008). Canonical mitochondrial localization is achieved by an amino terminal mitochondrial targeting leader sequence in the amino acid sequence that associates with subunits of the TOM complex (Yamano et al., 2008). Cooperation of TOM and TIM complexes allows for translocation of the presequence containing proteins to the mitochondrial matrix (Chacinska et al., 2004; Geissler et al., 2002; Yamamoto et al., 2002) where the presequence is cleaved by MPP (Taylor et al., 2001). The presence of this mitochondrial targeting sequence can be masked by the presence of other amino-terminal features, the cleavage of which allows for regulation of mitochondrial import (Mitra et al., 2007). Alternative mechanisms for mitochondrial import rely on carrier

proteins to associate transported proteins with import machinery or recognition of structural elements within the translocated protein (Bolender et al., 2008).

Analysis of the amino terminal mitochondrial targeting sequences of known mitochondrial proteins has yielded parameters that can be used to predict the potential of mitochondrial localization of unknown proteins based on the amino acid sequence of the protein of interest (Elstner et al., 2009; Emanuelsson et al., 2007). These tools (MitoP2 and TargetP) allow for rapid analysis of multiple sequences for amino-terminal MTS. The Maestro algorithm incorporates computational analysis with proteomic data to assign scores indicative of mitochondrial localization based on integrative analysis of the presence of an MTS, common protein domains, proteomic analysis by tandem-mass spectrum identification, as well as co-expression and transcriptional co-regulation (Calvo et al., 2006). Each tool is strictly predictive and as such the results require experimental validation.

Structural prediction based on the amino acid sequence utilizes the fundamental tendencies of amino acids to adopt conformations that promote formation of secondary structural elements (Cole et al., 2008). Despite significant amino acid substitutions, secondary structural elements can be preserved based on the ability of individual amino acids to adopt similar conformations. While the domain architecture of protein can be preserved, the overall structure of a protein provides insight into the catalytic mechanism. By comparing secondary structural elements of a protein of unknown structure to proteins containing similar secondary structural elements for which molecular structures have been determined, an approximate molecular structure can be determined (Kelley, 2009).

Results

The primary amino acid sequences of the 8 human homologues of AlkB were analyzed by the TargetP and MitoProt algorithms. The Maestro database was searched for the AlkB homologues. The results are summarized in Table 2.1. TargetP analysis predicts AlkBH3, 5 and 7 to be mitochondrial. Only AlkBH7 demonstrates a reliability class score of 2, which indicates a 0.6 to 0.8 probability of subcellular sorting to one compartment greater than to the next highest. Only AlkBH7 has a significant probability of mitochondrial localization according to MitoProt analysis. Integrative bioinformatic and proteomic analysis, as reported by Maestro score (range -12 to 42), indicates that, of the AlkB homologues represented, only AlkBH7 scores positively with a score of 23. The presence of canonical amino-terminal mitochondrial targeting sequences was evaluated by the MitoProt algorithm. The amino terminus of AlkBH7 is predicted to contain an MTS which is cleaved after 21 amino acids. Given this cleavage, the mature protein would have a molecular weight of 22.4 kDa.

TargetP analysis also considers the presence of signaling peptides for the secretory pathway (SP). None of the AlkB homologues show significant scores for secretion by this analysis.

Analysis of the secondary structural elements predicted to occur in the amino acid sequence of AlkBH7 as compared to AlkB, AlkBH1, AlkBH2 and AlkB3 is shown in Figure 2.1. The individual amino acid sequences were analyzed for the presence of secondary structural elements using Jpred. Alignments were manually annotated to demonstrate similar α-helix and β-sheet content. Overall structural elements are

Protein	Reference Sequence	Target P						MitoProt	Maestro (Mouse)
		Length	mTP	SP	other	Loc	Reliability Class		
AlkBH1	NP_006011	389	0.316	0.120	0.486	_	5	0.0679	NR
AlkBH2	NP_001001655	261	0.108	0.090	0.842	_	2	0.0509	-7
AlkBH3	NP_631917	286	0.706	0.048	0.255	M	3	0.0344	-1
AlkBH4	NP_060091	302	0.063	0.096	0.929	_	1	0.2374	-6
AlkBH5	NP_060228	394	0.537	0.033	0.525	M	5	0.0387	-4
AlkBH6	NP_116267	266	0.096	0.056	0.842	_	2	0.0195	-6
AlkBH7	NP_115682	221	0.727	0.063	0.126	M	2	0.8605	23
AlkBH8	NP_620130	238	0.191	0.075	0.826	_	2	0.0090	-5

NR- Not Represented

Table 2.1 Analysis of Human AlkB Homologs for Potential Mitochondrial Localization

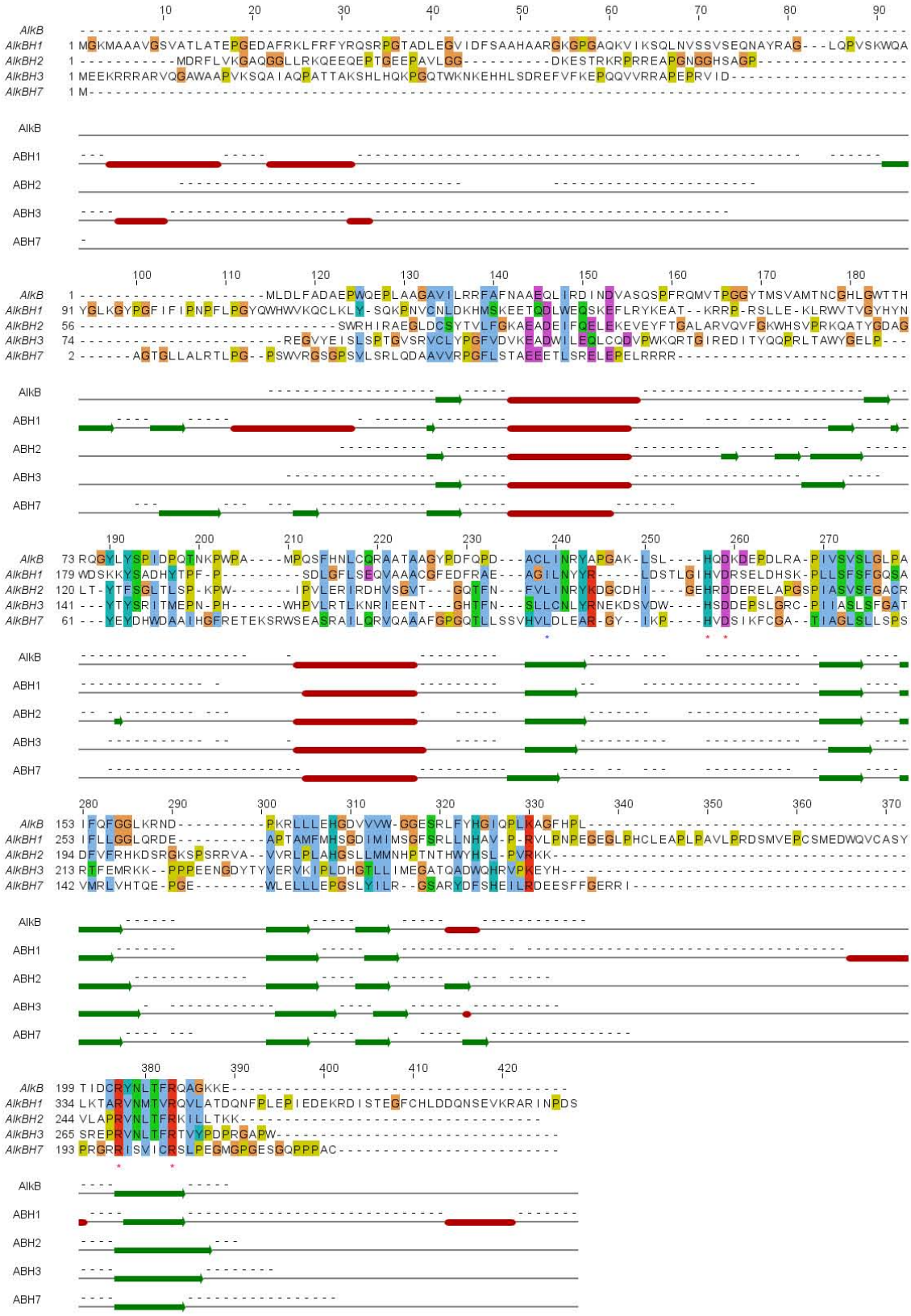

AlkB
AlkBH1
AlkBH2
AlkBH3
AlkBH7
ABH1
ABH2
ABH3
ABH7

Figure 2.1 Multiple Sequence Alignment of AlkBH7 with other Active AlkB Family Members. Full amino acid sequences were aligned using ClustalX. Secondary structure prediction used Jpred and alignments were refined manually in Jalview. ClustalX coloring shows amino acids with similar chemical properties. Conserved iron-binging residues are labeled with *, oxidized leucine necessary for α-KG orientation in the active site is labeled with *.

conserved among the family including iron binding residues His 131, Asp 133, Arg 203 and Arg 209 in AlkB which are conserved in AlkBH7 as His 121, Asp 123, Arg 197 and Arg 203. A unique feature of the AlkB family is the presence of a partially oxidized leucine at position 177 of AlkBH3 (Sundheim et al., 2006). This AA is conserved in AlkB, AlkBH2 and AlkBH7 at positions 118, 157 and 110, respectively. In AlkBH1, the leucine is replaced by isoleucine at position 218. This oxidized leucine is responsible for orienting α-KG in the active site based on a co-crystal of AlkBH3 with Fe^{2+} and α-KG. Replacement of this leucine with glutamine results in a catalytically inactive protein (Sundheim et al., 2006).

Discussion

In order to predict if any of the human AlkB homologs could contribute to mtDNA maintenance by direct repair, the protein sequences of all 8 human AlkB homologues were input into Target P and MitoProt algorithms for mitochondrial prediction. The output was compared to the Maestro dataset for mouse proteins. As shown in Table 2.1, MitoProt analysis yielded 3 homologs, AlkBH4, AlkBH7 and FTO with potential mitochondrial localization. Target P analysis predicted mitochondrial localization for AlkBH3, AlkBH5 and AlkBH7. Of these, only AlkBH7 has a substantial difference in subcellular localization potential as indicated by "reliability class." As a Class 2 result, the probability of mitochondrial localization is between 0.6 and 0.8 greater than other subcellular targeting prediction. As the common result of these searches, the Maestro database was searched for the mouse homolog of AlkBH7. The Maestro score for AlkBH7 of 23 is significant as other proteins associated with transcription, replication

and repair of the mtDNA score lower. Significantly, TFAM has a Maestro score of 13, DNA Pol γA a score of 4, and mTERF a score of 3.

Analysis of the secondary structure of AlkBH7 suggests that it contains similar elements to other family members which have been shown to be active direct repair enzymes. Predicted structural homology between AlkBH7 and AlkB, AlkBH1, AlkBH2 and AlkB3 is significant; however there are elements of significant difference between the individual proteins. Each amino acid sequence contains a unique amino terminus which has little homology to other family members and features relatively few secondary structural elements. In the case of AlkBH7, this amino-terminal leader sequence contains 32 AA prior to the beginning of the first predicted β–sheet common among the family members. It is within these amino acids that the MTS is predicted to occur, thus if AlkBH7 were to be imported to the mitochondria and the targeting sequence cleaved, the core of the protein would be unaffected. Unlike AlkB, AlkBH1, AlkBH2 and AlkBH3, the α–helices of AlkBH7 are not separated by predicted secondary structural elements. When comparing the available crystal structures for AlkB, AlkBH2 and AlkBH3, these inter-helical sequences provide a cap on the end of the protein potentially limiting access to the active site (Figure 2.2). This inter-helical domain in AlkBH2 forms a long loop which contributes to flipping adducted bases into the active site by inserting a phenylalanine into the DNA strand to stabilize the helix during catalysis (Yang et al., 2009a). Lacking this long loop structure, it is proposed that AlkB, and presumably AlkBH7, function slightly differently in that the mechanism of base flipping in AlkBH2 closely resembles that of DNA glycosylases while AlkB compresses the helix of the lesion-containing DNA strand resulting in the damaged base flipping into the active site

(Yang et al., 2009a). Despite these differences, the domain architecture of AlkBH7 closely resembles other family members, inferring that it can adopt the β- jellyroll structure of the family and potentially display catalytic activity as a direct reversal DNA repair protein.

While not predicted to have a strong amino-terminal MTS, AlkBH1 has been shown to localize to the mitochondria of HeLa cells experimentally (Westbye et al., 2008). In an alternative report, expression of AlkBH1 in mouse embryo fibroblasts is shown to be nuclear, presumed to interact with euchromatin and possibly to have a role in epigenetic regulation of gene expression (Pan et al., 2008). In conjunction with these reports, TargetP analysis of AlkBH1 shows a significant possibility of mitochondrial targeting (0.316) while also maintaining the possibility of alternative subcellular localization (0.486.) These disparate experimental results illustrate the predictive nature of the analysis conducted in this study. Alternatively, the observations of AlkBH1 localization could be attributed to the differences in the cell types studied or other experimental variations.

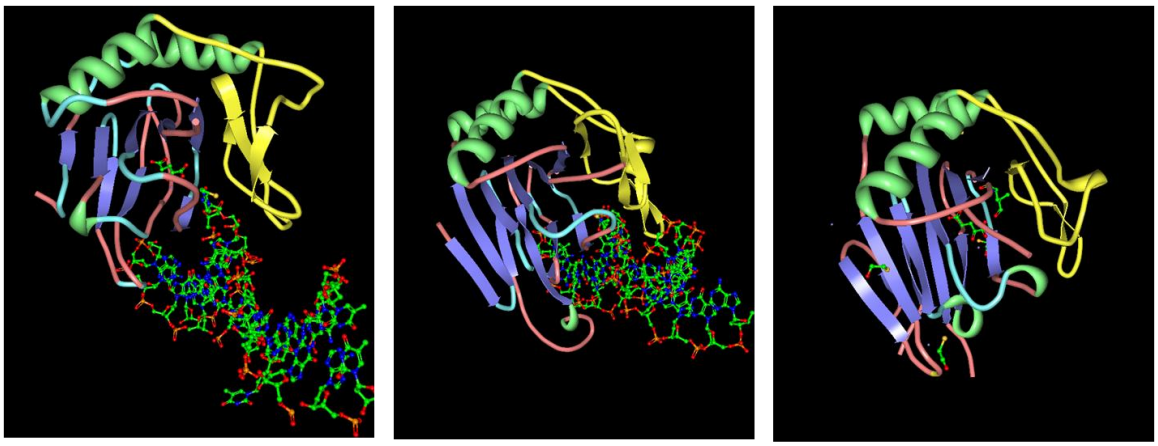


Figure 2.2 Molecular Structures of AlkB, AlkBH2 and AlkBH3. The crystal structures of AlkB in complex with dsDNA, AlkBH2 in complex with dsDNA and AlkBH3 available from PDB (accession numbers indicated below each structure) were colored similarly. Green structures are α-helices, β- strands are blue. The yellow color is used to highlight the amino acid loops between the α-helices which are common among these AlkB family members but are truncated in AlkBH7.

Chapter 3: Subcellular Localization of AlkBH7

Introduction

The mitochondrial proteome consists of between 900 and 1500 proteins. The human mitochondrial genome encodes only 13 proteins. The remaining mitochondrial proteins are encoded in the nuclear genome and are imported into the mitochondria. These proteins include major components of the electron transport complexes as well as those which allow for efficient replication, repair, and transcription of the mtDNA, processing and translation of mRNAs as well as other critical mitochondrial functions.

The mitochondrial genome is associated with a set of proteins defining an ultrastructure termed the mitochondrial nucleoid (Albring et al., 1977; Bereiter-Hahn, 1996; Bogenhagen et al., 2008; Bogenhagen et al., 2003; Garrido et al., 2003). The proteinaceous component of the mitochondrial nucleoid is believed to offer protection from spontaneous oxidation of the DNA as well as to provide a scaffold for regulation of mtDNA-associated function. Transcription factor A mitochondrial (Tfam) and mitochondrial single stranded binding protein (mtSSB) associate tightly with the mtDNA (Bogenhagen et al., 2003; Ghrir et al., 1991; Li, 1997). Other proteins associated with the mtDNA fall into general groups associated with DNA binding and metabolism, chaperones and other metabolic and structural proteins (Wang, 2006). Each discrete nucleoid is proposed to contain 5-7 copies of the mtDNA (Iborra et al., 2004; Legros et al., 2004).

If AlkBH7 contributes to the maintenance of mtDNA, it would be expected that the protein would associate with mtDNA in cells. As predicted by the informatic analysis, the amino terminus of the protein should be sufficient to target the protein to the mitochondrial matrix. In order to test this prediction, the ability of AlkBH7 to localize with the mitochondria was tested by fluorescence microscopy and co-fractionation of the protein with mitochondria purified by differential centrifugation.

Materials and Methods

Subcellular Localization by Fluorescence Microscopy

Analysis of the Predicted MTS of AlkBH7

A cassette encoding the amino terminal 17 amino acids of AlkBH7 was amplified from human testis cDNA (Clontech) using PCR primers with the sequences 5'-GGAATTCATATGGCCGGGACTGGGC-3' and 5'-GCGGATCCGTGCTCAGGAAGCCAG-3'. The product was cloned into pCR-Blunt II-TOPO and sequenced. The EcoRI/ BamHI fragment was then subcloned into pM-DSRed, a derivative of pDsRed1-N1 developed in our laboratory to permit in-frame cloning of putative mitochondrial targeting signal sequences (Perez-Jannotti et al., 2001). The construct was transfected into HeLa cells using Fugene 6.0 Transfection Reagent according to the recommended protocol. 48 hours later, 20 nM Mitotracker Green (Invitrogen) was added for 30 min to label mitochondria and cells were imaged using fluorescence microscopy to detect Mitotracker Green fluorescence and DsRed fluorescence using standard fluorescein and rhodamine filter sets in the Zeiss LSM510 confocal microscope.

Association of full length AlkBH7 with Tfam

To express an epitope-tagged version of the full length protein, AlkBH7 was amplified from testis cDNA by PCR using primers with the sequence 5'-GGCATATGGCCGGGACTGGGC-3' and 5'-TGCTCGAGGCAGGCTGGAGGCGGCTG-3'. After cloning into pCR-Blunt II-TOPO and sequencing, the NdeI/XhoI cassette was subcloned into pCDNA4/TO/myc-hisA. The construct was transfected into HeLa cells using Fugene 8 transfection reagent. After 48 hours, the cells were fixed and permeabilized. AlkBH7 was detected using a monoclonal antibody against the myc epitope tag and AlexaFluor 568-labeled goat anti-mouse secondary antibodies. Tfam was detected using a polyclonal antibody and AlexaFluor 488-labeled goat-anti-rabbit secondary antibodies. Imaging of the fluorescence signals was done on a Zeiss 510 meta confocal microscope.

Purification of mitochondria from cells expressing AlkBH7-myc-his

HeLa cells were grown in DMEM supplemented with 5% (v/v) calf serum and 5% (v/v) fetal bovine serum with penicillin and streptomycin to inhibit microbial growth. HeLa cells were transfected with the full length expression construct described above. After 24 hours, transfected cells were selected with 400 μg/ml Zeocin for an additional 48-72 hours, after which cells were split 1:10 into fresh media containing 200 μg/ml Zeocin. During purification of mitochondria, all handling was at 4°C. All buffers contained the protease inhibitors PMSF, Pepstatin A, Leupeptin and E64. Cells were harvested by scraping plates with a rubber policeman and collected in PBS. Cells were pelleted at 500 x g for 5 min, resuspended in hypotonic buffer (20 mM HEPES pH 8, 5

mM KCl, 1.5 mM $MgCl_2$, 2 mM DTT) and repelleted by spinning at 900 x g for 5 min. The cells were resuspended in 9 ml of hypotonic buffer and homogenized with 10-15 strokes of a glass Dounce homogenizer. 6 ml of 2.5X MSH (525 mM mannitol, 175 mM sucrose, 50 mM HEPES, pH 8, 5 mM EDTA), were added per 9 ml. Nuclei were removed by two successive centrifugation steps for 5 min at 1500 g. The post nuclear supernatant (PNS) containing the mitochondria was centrifuged for 15 min at 13,000 rpm to pellet the crude mitochondria (CM).

The crude mitochondria then were resuspended in 0.5 ml 1X MSH and layered on a 0.8 M/1.5 M preformed sucrose step gradient in a Beckman SW41 tube. Gradients were centrifuged at 23,000 rpm at 4 °C for 30 min. Mitochondria, which sediment to the 0.8 M/1.5 M sucrose interface, were collected with a sterile Pasteur pipette. The mitochondria were diluted with 4 volumes of 1X MSH, re-pelleted at 13000 rpm and resuspended in 0.5 ml 1X MSH. Protein content was determined using the Bradford dye binding assay as above. 50 to 100 μg aliquots of mitochondria were pelleted and frozen at -80°C.

Results

While informatic analysis of AlkBH7 suggests that the amino-terminal 21 AA of AlkBH7 may contain the mitochondrial targeting sequence, the cleavage of leader sequences directing mitochondrial import is rarely exact. The N-terminal 17 AA were fused to red fluorescent protein (RFP) to determine whether this putative signal sequence was sufficient to target the protein to mitochondria. As shown in Figure 3.1, panel A, the amino terminal 17 AA of AlkBH7 are sufficient to drive mitochondrial targeting of RFP

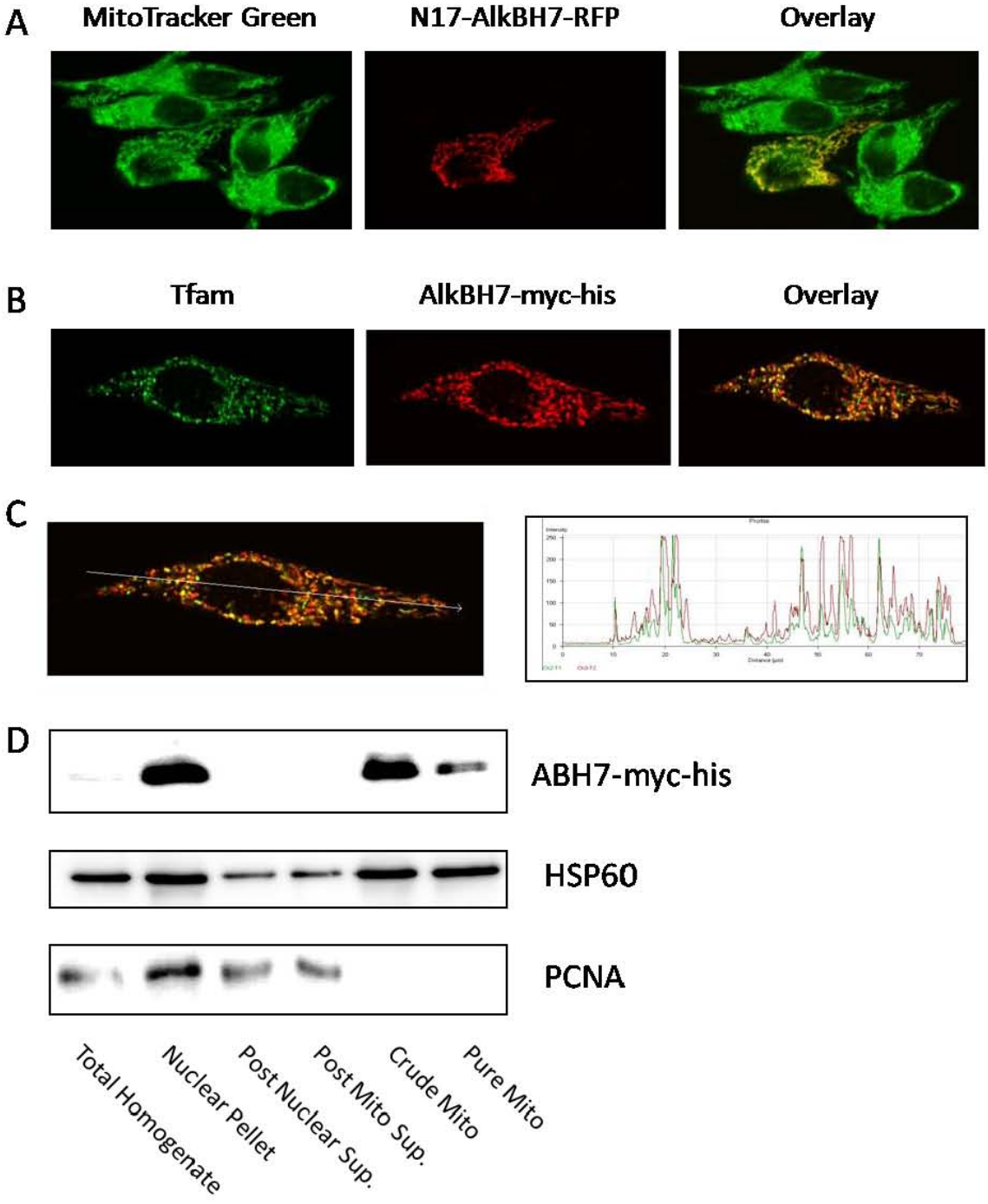

A
MitoTracker Green
N17-AlkBH7-RFP
Overlay
B
Tfam
AlkBH7-myc-his
Overlay
C
D
ABH7-myc-his
HSP60
PCNA
Total Homogenate
Nuclear Pellet
Post Nuclear Sup.
Post Mito Sup.
Crude Mito
Pure Mito

Figure 3.1 Subcellular localization of AlkBH7. **A:** Mitochondrial targeting of RFP by the N-terminal 17 AA of AlkBH7. A construct expressing the first 17 AA of AlkBH7 fused in-frame to RFP was constructed and transfected into HeLa cells. 48 hours after transfection, cells were labeled with Mitotracker Green and visualized by fluorescence microscopy. B: Colocalization of AlkBH7 with Tfam. Full-length AlkBH7 was expressed with a C-terminal myc-his epitope tag. Endogenous Tfam and transgenic AlkBH7-myc-his were detected using rabbit polyclonal antibodies against Tfam and mouse monoclonal antibodies against c-myc. Secondary antibodies coupled to Alexa fluorescent dyes were used to visualize primary antigens. C: Analysis of colocalization of Tfam with AlkBH7. Fluorescence signals for Alexa 488 and 525 were analyzed along the line indicated in the left panel. Histogram of fluorescence signal intensities for both 488 and 525 emissions. D: Fractionation of AlkBH7 with the mitochondrial fraction during purification by differential centrifugation.

as evidenced by colocalization with Mitotracker Green. The red fluorescent signal observed shows ribbon-like patterning and is excluded from the nucleus. These characteristics are shared by many mitochondrially associated proteins. Mitotracker Green fluorescence is slightly more diffuse but shows a similar ribbon-like mitochondrial distribution. The overlay indicates that in transfected cells, AlkBH7MTS-RFP colocalizes with the mitochondria.

If AlkBH7 contributes to maintenance of the mitochondrial genome, it would be expected that the full length protein would associate with mtDNA containing structures. A DNA construct allowing for the expression of full length AlkBH7 with a C-terminal myc-his epitope tag was created and transfected into HeLa cells to allow for detection of the protein using immunofluorescence microscopy. Anti-sera raised against Tfam which localizes strongly with mtDNA was used to identify nucleoids. Figure 3.1, panel B shows a typical result of these colocalization experiments. Tfam localizes to punctate structures in the cytoplasm indicative of nucleoid structures. AlkBH7-myc-his colocalizes with these structures indicating a close physical association of full length AlkBH7 with mitochondrial nucleoids. As shown in the lower panel of Figure 3.1, panel C, fluorescence signals for both Tfam and AlkBH7-myc-his were highly coincident although relative intensity varied between individual spots. These results are consistent with a general nucleoid association, but higher resolution imaging methods would be required for a definitive analysis.

To determine whether tagged AlkBH7 copurified with the mitochondria, HeLa cells transfected with the epitope tagged full-length AlkBH7 expression construct were cultured and the mitochondria isolated by differential centrifugation. Analysis of the

proteinaceous material fractionated in this way shows that AlkBH7-myc-his segregates with the mitochondrial chaperone Hsp60 while PCNA is enriched in the nuclear pellet (Figure 3.1, panel D.)

Discussion

The mitochondrial proteome consists of between 900 and 1500 nuclear encoded proteins that are transported to the mitochondria following translation. Canonical mitochondrial import is determined by an amino-terminal leader sequence featuring an amphipathic helix. Analysis of the N–terminal sequence of AlkBH7 in Chapter 3 suggested it would encode a mitochondrial targeting signal (MTS).

Tfam associates with the mtDNA and packages mtDNA into nucleoid like particles (Alam et al., 2003; Kaufman et al., 2007). Immunoprecipitation with Tfam has been used to characterize the proteins associated with the mtDNA (Bogenhagen et al., 2003; Wang, 2006). As AlkBH7 is predicted to be a mitochondrial protein capable of contributing to maintenance of the mtDNA, the association of protein with mtDNA would be expected. Tfam was used as a marker for the mitochondrial nucleoids. The full-length of AlkBH7 is shown to associate with TFAM forming punctate cytoplasmic structures. These discrete structures are presumed to contain mtDNA in vivo and localize to discrete areas of the mitochondria. Mitochondrial DNA glycosylases UDG1, mitochondrial MYH, NTH1 and OGG1 have been shown to localize discretely to the mitochondrial nucleoid although MYH and NTH1 have been shown to localize to the mitochondrial matrix (Ikeda et al., 2002; Ohtsubo et al., 2000). Uracil excision, 8-oxoguanine excision, and AP endonuclease activities co-segregate with a particulate fraction of the inner mitochondrial membrane (Stuart et al., 2005). AlkBH7 segregated

with TFAM in crude biochemical separations of mitochondria from HeLa cells verifying that the observed co-localization is preserved in vivo. Submitochondrial fractionation and association of the AlkBH7 with purified nucleoids would be required to demonstrate nucleoid association; however the inference of nucleoid localization is strong based on the data presented.

Proteomic analysis of mitochondrial nucleoids has not detected DNA repair proteins associated with the mtDNA (Bogenhagen et al., 2008; Wang, 2006). This can be attributed to the low abundance of mitochondrial DNA repair proteins. It is interesting to speculate that some DNA repair proteins may localize to nucleoids in a more definitive manner following DNA damage. Interestingly DNA2, a helicase associated with mtDNA repair shows increased nucleoid localization as a consequence of stalled replication (Duxin et al., 2009). Mitochondrial BER activity has been shown to be up-regulated after ischemia/reperfusion in rat brain (Chen et al., 2003), in the development of Parkinson's Disease (Arai et al., 2006) and as a consequence of aging in the liver (de Souza-Pinto et al., 2001b). These biochemical descriptions of up-regulated DNA repair activity suggest that either mitochondrial DNA repair proteins interact transiently with the DNA or that basal levels of DNA damage are insufficient to stimulate association of repair factors with the mitochondrial nucleoid. The general association of AlkBH7 with TFAM suggests that when expressed, AlkBH7 is capable of associating with mitochondrial nucleoids. This allows for the possibility that AlkBH7 expression is controlled in either a tissue specific or stress response fashion but cannot exclude the possibility that both potential mechanisms contribute to regulation. As proteomic studies of mitochondrial nucleoids are undertaken, it will be critical to evaluate the proteins associated with both

normal and stressed nucleoids to fully understand the nature of the proteins that contribute to maintenance of the mtDNA.

Chapter 4: AlkBH7 Repairs DNA Damage by a Direct Reversal Mechanism

Introduction

AlkB is capable of catalyzing the direct removal of methyl- groups from 1-methyladenine, 3-methylcytosine, 3-methylthymine and higher alkylations such as ethano- and etheno- bridges between the N^1 and N^6 atoms of adenine (Delaney et al., 2005; Falnes et al., 2002; Frick et al., 2007; Trewick et al., 2002). The mechanism employs Fe^{2+}, α-ketoglutarate and O_2 in a hydroxylation reaction (Trewick et al., 2002). The DNA adduct is removed as formaldehyde in the case of methylations (Trewick et al., 2002) and glyoxal in the case of etheno lesions (Delaney et al., 2005). Evidence indicates that ethano lesions are not completely reversed but proper Watson-Crick hydrogen bonding is restored by the hydroxylation (Frick et al., 2007).

As shown in the previous chapter, AlkBH7 localized to the mitochondria. Since the amino terminal 17 amino acids encode a sufficient MTS, a mature protein which has been imported to the mitochondria could be cleaved by MPP between the 17^{th} and 33^{rd} amino acids without affecting conserved secondary structure elements. While the mature N-terminus of AlkBH7 has not been determined, the fact that 17 AA are sufficient to direct the protein to mitochondria suggests that it is unlikely that a much larger segment of the protein is excised. It therefore is fair to expect that a mature mitochondrial AlkBH7 protein could contain all structural elements downstream of the 17 AA leader sequence.

Exocyclic DNA lesions vary widely in their size and chemistry. Methylations represent the smallest class of DNA lesions and can be generated endogenously by aberrant SAM methylation or by exogenous chemotherapeutic compounds. Large exocyclic DNA adducts such as benzo[a]pyrene can intercalate into DNA and disrupt replication or transcription. While a wide range of processes are available to mitigate the effects of exocyclic DNA lesions in nuclear DNA, mitochondria do not posses a full complement of these repair processes. Substituted and unsubstituted etheno lesions represent a small subset of moderately sized yet potentially mutagenic exocyclic DNA adducts that can arise from either endogenous or exogenous sources. MtDNA is subject to many of the same stresses that affect the nuclear genome, yet repair mechanisms for alkylations and LPO adducts have not been characterized. As AlkBH7 is imported to the mitochondria and associates with the mtDNA containing nucleoids, the ability of AlkBH7 to catalyze the direct removal of methyl groups from 1-meA, 3-meC and εA was tested to evaluate the potential contribution of AlkBH7 to maintenance of the mtDNA.

Materials and Methods

Materials

All chemical reagents were obtained from Sigma Aldrich (St. Louis, MO). Oligonucleotides used for cloning were purchased from Qiagen (Valencia CA.) Oligonucleotides used as biochemical substrates were synthesized in the Stony Brook University Department of Pharmacology. AAG was purchased from Trevigen, Inc (Gaithersburg, MD.)

Molecular Cloning and Protein Purification

AlkB was cloned from E. coli genomic DNA by PCR using primers 5'-GGCATATGTTGGATCTGTTTGCCGATGC-3' and 5'-AAGACTCTTCCTTCTTACCTGCCTGACG-3'. The amplified cassette was cloned into pCR-Blunt II-TOPO (Invitrogen) and sequenced. The NdeI/XhoI cassette was subcloned into pET22b$^+$ to create a construct that allowed expression of AlkB-6xHis. Protein was expressed in BL21 (DE3) pLysS E.coli overnight at 18° C in the presence of 0.3 mM IPTG in 2xYT media. The culture was centrifuged and supernatant removed. The E. coli were resuspended in a lysis buffer containing 50 mM HEPES, pH 8.0, 300 mM NaCl, 5% glycerol, 0.5% TX-100, 1 mM β-mercaptoethanol and supplemented with 5 μg/ml aprotinin, 5 μg/ml leupeptin and 0.2 mM PMSF. Cells were lysed by sonication and insoluble material was removed by centrifugation at 30,000xg for 40 min. The protein containing supernatant was then loaded onto a 5 ml HisTrap nickel affinity column and washed with 5 column volumes of lysis buffer supplemented with 50 mM imidazole. The protein was eluted using a linear gradient from 50 mM to 1 M imidazole and collected in 1 ml fractions. Fractions containing AlkB-his were identified by SDS-PAGE, pooled and diluted into 2X volume of 20 mM PIPES, pH 6.3, 5 mM EDTA, 2 mM β-mercaptoethanol. The diluted fractions were applied to a Mono S ion exchange column from which AlkB-his protein was eluted during a linear gradient from 10 mM NaCl to 1 M NaCl in the above buffer.

An expression cassette for the mature form of AlkBH7 (mAlkBH7) was created similarly to that of AlkB using PCR primers: 5'-CCATATGCGAGGCTCGGGCCCTTCC-3' and 5'-TGCTCGAGGCAGGCTGGAGGCGGCTG-3' on HeLa first strand cDNA (Clontech).

Expression and lysis were done similarly to expression for AlkB-6xHis except that BL21 (DE3) pLysS RIPL cells were used for expression. The lysis buffer for mAlkBH7-6xHis consisted of 50 mM HEPES, pH 8.0, 300 mM NaCl, 25 mM imidazole, 5% glycerol, 0.5% TX-100 with protease inhibitors. The cleared, filtered lysate was applied to a 5 ml HisTrap nickel affinity column. The column was washed with 10 column volumes of lysis buffer adjusted to 1 M NaCl then washed with 10 column volumes of lysis buffer adjusted to 25 mM NaCl before elution with a 15 column volume linear gradient from 25 mM imidazole to 1M imidazole. Fractions containing mAlkBH7-6xhis were identified by SDS-PAGE and pooled. Pooled fractions were dialyzed into 25 mM MES, pH 6.0, 2 mM NaCl, 5% glycerol, 1mM EDTA, 3 mM β-mercaptoethanol, 0.1% TX-100 supplemented with protease inhibitors. The dialyzed protein was filtered and applied to a Mono S ion exchange column from which it was eluted using a linear gradient from 2 mM NaCl to 1M NaCl in the above buffer.

Site directed mutagenesis of the mature form of AlkBH7-6xhis in pET22b+ was conducted using extension primers: 5'- GTGCACGTGCAGGGACCTGGAA-3' and TTCCAGGTCCTGCACGTGCAC-3' to create an expression construct containing a Leu to Gln substitution in the active site of AlkBH7. The construct was expressed in BL21 (DE3) pLysS RIPL and purified using identical conditions to AlkBH7-6xhis.

Demethylase Activity of AlkB and mAlkBH7

Oligonucleotides containing 1-methyldeoxyadenine or 3-methyldeoxycytidine within a Dpn II restriction sequence were prepared by the Department of Pharmacology DNA synthesis facility (Table 3.1). Lesion-containing oligomers and the control oligomer were radiolabeled on the 5' end using T4 polynucleotide kinase and γ-^{32}P-ATP.

Labeled oligomers were separated on a 15% PAGE-urea gel and purified. The oligomers were annealed to a 1.25-fold molar excess of their complementary strands in a buffer containing 10 mM Tris, pH 8.0, 50 mM NaCl and 2 mM $MgCl_2$ by heating the oligonucleotides to 90 °C for 10 min then cooling slowly over 4 hr. Oligos were diluted 1:10 for biochemical assays. Demethylase activity of AlkB and AlkBH7 was tested by incubating the oligonucleotides with purified protein for 60 min at 37 °C in a reaction that contained 50 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 1 mM α-ketoglutarate, 75 μM $FeCl_2$, 2 mM ascorbic acid and 50 μg/ml BSA in a volume of 20 μl. After 60 min, Dpn II Buffer, Dpn II enzyme and water were added to a final volume of 50 μl. The restriction digestion was incubated at 37 °C for 60 min. The DNA was ethanol precipitated and resuspended in 7.5 μl TE to which 7.5 μl of 2x formamide loading solution was added before being heated to 80° C for 5 minutes and loaded onto a pre-run 15% PAGE-urea gel. The gel was exposed to a phosphor screen and imaged using a Storm Phosphorimager (GE Healthcare).

AAG Cleavage Protection Assay (Glycosylase Sensitivity Assay)

A 62-mer oligonucleotide containing a single $1,N^6$ ethenodeoxyadenosine residue (Table 3.1) was labeled, gel purified and annealed as described above. The annealed substrate was incubated with purified AlkB or AlkBH7 for 60 min at 37 °C in the reaction buffer described above. Following the primary reaction, 5 μl of AAG reaction buffer, 1 μl of AAG (Trevigen, 1.5 U/μl) and 24 μl of water were added to each tube. The reactions were incubated at 37 °C for 60 min. Following glycosylase treatment, NaOH was added to a final concentration of 100 mM and the reactions were heated to

Oligo	Length	Sequence 5'-3'	Assay
Control	49	ATTGCCAAGCTTCGATAGGATCCGGTCAAACCTAGACG AATTCGTAGAC	DpnII
1-meA	49	ATTGCCAAGCTTCGATAGG-1meA-CCGGTCAAACCTAGACGAATTCGTAGAC	DpnII
3-meC	49	ATTGCCAAGCTTCGATAGGAT-3meC-GGTCAAACCTAGACGAATTCGTAGAC	DpnII
Complement	49	GTCTACGAATTCGTCTAGGTTTGACCGGATCCTATCGA AGCTTGGCAAT	DpnII
εA-62	62	TGCGAAGCTTCGATACCACTCGGCCTT-εA-TCTACTTTCCTCTCCATTTGAGCGGTCAGGAGCT	GSA
Complement-62	62	AGCTCCTGACCGCTCAAATGGAGAGGAAAGTAGATAA GGCCGAGTGGTATCGAAGCTTCGCA	GSA
εA-18	18	GATATCT-εA-TTACACTTTA	MS

Table 4.1: Oligonucleotides Used to Characterize Direct Repair Activity of AlkBH7

85° C for 10 min. Finally, DNA was ethanol precipitated and resuspended in 7.5 μl of TE. Samples were analyzed on 15% PAGE-urea gels as described above.

Fluorescence Detection of εA removal by AlkBH7

A Horiba Jobin Yvon Fluorolog 3 controlled by SpectraAcq software was employed to detect the fluorescent signature of εA. Monochromatic excitation at 315 nm was employed. Emission spectra from 380 to 440 nm at 1 nm resolution were collected. Peak emission of εA was observed at 406 nm. Reactions were done in 300 μl volume at 37 °C in conditions described above. DNA substrate was present at 228 nM while enzyme concentration for both AlkB and AlkBH7 experiments was 135 nM.

Mass Spectrometric Analysis of Etheno Removal

400 pmoles of εA-18 (Table 4.1) was incubated in AlkB reaction buffer as above in the presence or absence of AlkB-6xHis or AlkBH7-6xHis in 150 μl volume at 37 °C for 2 hours. Following the incubation, the reactions were phenol-chloroform extracted twice. Residual phenol-chloroform was removed by ether extraction. The oligo was then ethanol precipitated and dried. Samples were dissolved in 25 μL of 0.1M triethylammonium acetate (TEA), concentrated and desalted on C_{18} ZipTips (Millipore), washed with 0.1M TEA and eluted into 10 μl of 50% acetonitrile/water. The samples were then dried in a rotary evaporator (Savant), resuspended in 10 μl of 50% acetonitrile/water containing 0.1% triethylamine and infused at 5 μL/min into the ion source of a TSQ Quantum Access mass spectrometer (Thermo). The instrument was operated in the negative electrospray ionization mode and several scans were averaged to produce each spectrum. The raw spectra were further processed through the

ProMassXcali software (ver 2.5 SR-1) to determine charge state and to deconvolute the charge state distribution to the mass scale.

Results

In order to test the ability of AlkBH7 to catalyze the direct removal of DNA lesions, 2 different DNA constructs were generated to permit expression of the wild-type protein and a variant predicted to lack repair activity. Both constructs featured a 17 AA truncation on the amino terminus of the protein. As this sequence is sufficient to drive mitochondrial targeting, and is outside the predicted core catalytic domain of the protein, it was reasoned that this truncation would not affect the catalytic competency of the protein. Further, both constructs featured a carboxyl-terminal 6x His epitope to allow purification by nickel affinity chromatography. The difference between the constructs was an L110Q site-specific AA substitution predicted to give rise to a catalytically inactive protein. A similar construct encoding E. coli AlkB-6xhis was created for use as a positive control for enzymatic reactions.

The proteins were expressed in E. coli allowing for high levels of expression. Purification by nickel affinity chromatography was followed by dialysis to remove imidazole and change the pH. The dialyzed protein was applied to a Mono S column and eluted as a discrete peak at 400 mM NaCl. It was found to be necessary to decrease the pH of the mobile phase during cation exchange chromatography to facilitate binding of the AlkBH7 proteins to MonoS. Analysis of the protein by silver-stained SDS-PAGE gel shows that both AlkB-6xhis and ΔN17-AlkBH7-6xhis proteins migrate at the expected molecular mass and are free of contaminants (Figure 4.1).

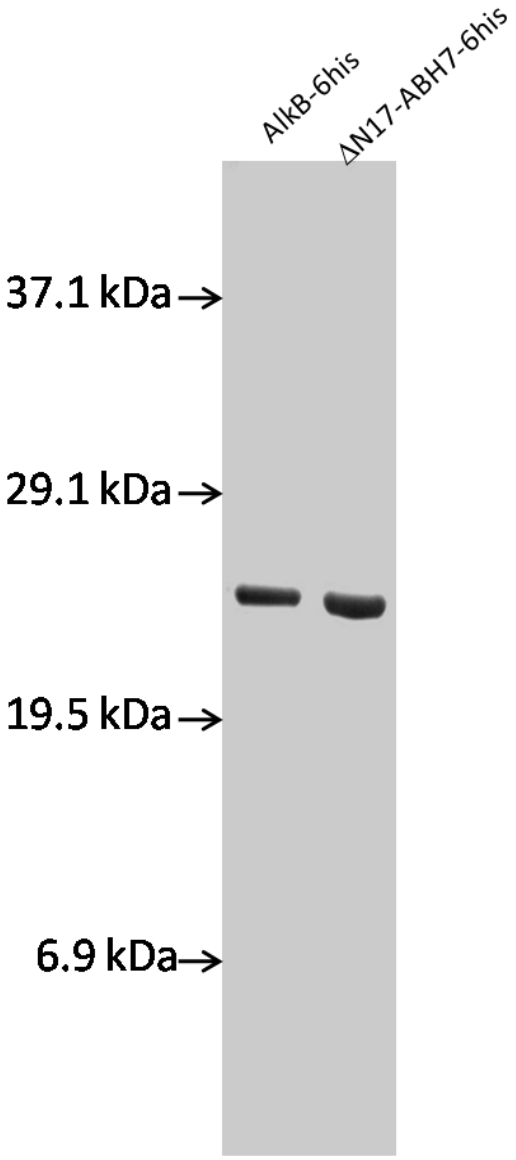


Figure 4.1: Purified AlkB-6his and ΔN17-AlkBH7-6his. Proteins expressed and purified as described in Materials and Methods were boiled in SDS loading buffer and subjected to discontinuous SDS-PAGE on a 15 % acrylamide gel. The gel was silver stained to visualize the proteins in each lane.

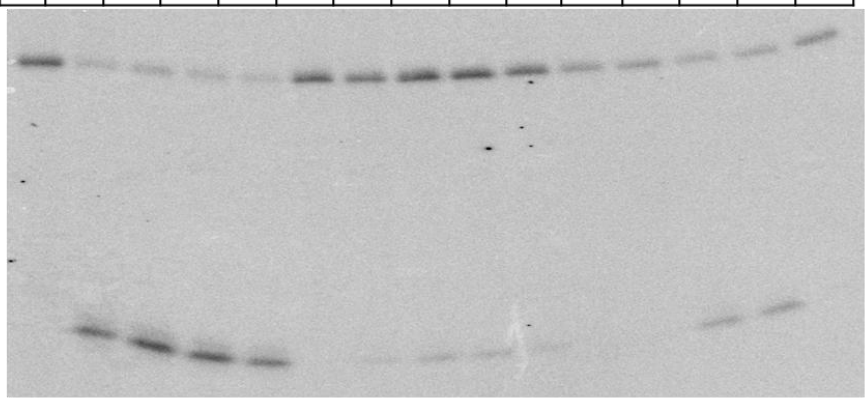
To assay for the ability of the purified protein to catalyze direct removal of methyl groups, an assay was employed which utilized the ability of 1-meA and 3-meC to block the DNA cleavage by DpnII. Utilizing a double-stranded oligonucleotide substrate set on which the lesion-containing strand is labeled with ^{32}P to allow for detection, the demethylation reaction was conducted prior to the addition of DpnII to the reaction. In this assay system, AlkB and AlkBH7 but not AlkBH7-L110Q demonstrated the capacity to remove the methyl group blocking DpnII cleavage on the 3-meC. A double-stranded oligonucleotide containing 1-meA was a poor substrate for all proteins tested (Figure 4.2).

LPO-mediated DNA lesions are likely to be formed in the mtDNA. Without expression of an alkyladenine glycosylase (AAG) to initiate BER of etheno- lesions in the mtDNA, the potential mutagenicity of these lesions could affect replication and repair of the mtDNA. The ability of AlkB, AlkBH7 and AlkBH7 L110Q to catalyze the direct repair of εA lesions from oligonucleotide substrates was assayed. A glycosylase sensitivity assay (GSA) was developed utilizing the ability of AAG to recognize and excise εA but not adenine bases from DNA creating an AP site. Subsequent enzymatic treatment with AP endonuclease or chemical hydrolysis under alkaline conditions was used to cleave the AP site resulting in a higher mobility labeled fragment of the original oligonucleotide. In this assay the starting material and the repaired product have a mass difference of 24 Daltons, a change that is not detectible on the PAGE-urea gels used to separate the products. The assay protocol is summarized in Figure 4.3 panel A.

Incubation of the purified proteins with the εA containing oligonucleotides and subsequent enzymatic/ chemical processing shows that both AlkB and AlkBH7 but not

A

	ssDNA														
Substrate	Control (A)					1-meA					3-meC				
AlkB/AlkBH7	-	-	B	7	Cl 7	-	-	B	7	Cl 7	-	-	B	7	Cl 7
DpnII	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+



B

	dsDNA														
Substrate	Control (A)					1-meA					3-meC				
AlkB/AlkBH7	-	-	B	7	Cl 7	-	-	B	7	Cl 7	-	-	B	7	Cl 7
DpnII	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+

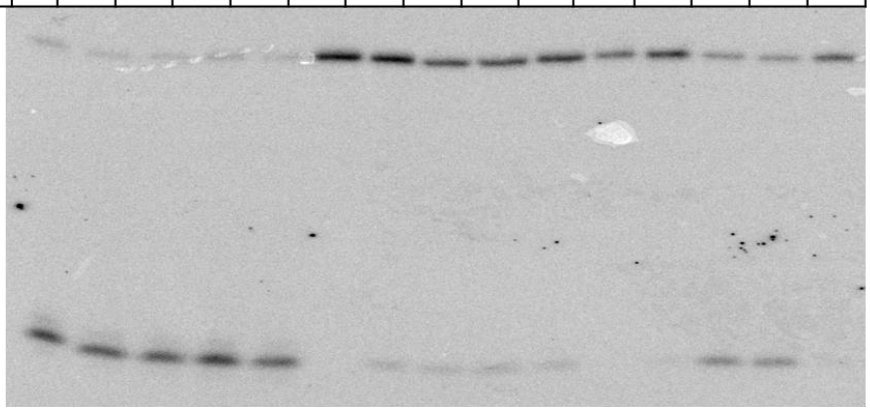
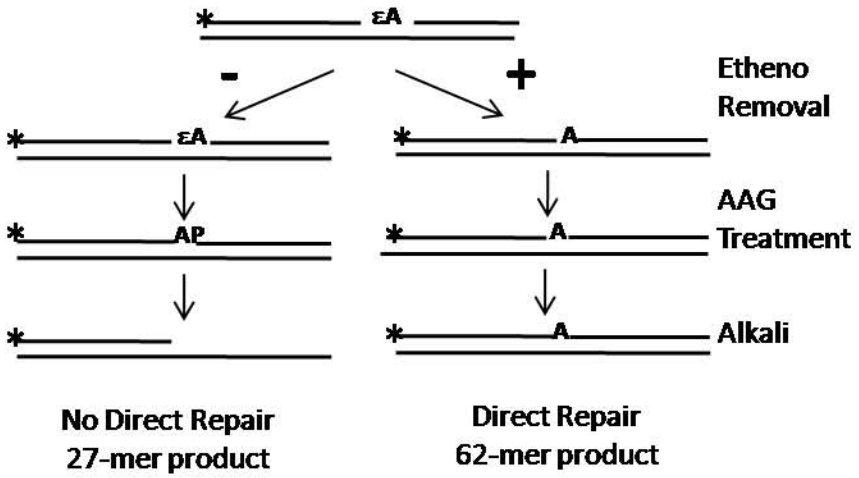


Figure 4.2: 3-meC but not 1-meA are repaired by AlkB/ AlkBH7 from single-stranded and double-stranded oligonucleotide substrates. 5'-^{32}P-labeled oligonucleotide substrates containing either 1-meA or 3-meC were incubated with AlkBH7 elution buffer, AlkB, AlkBH7 or catalytically inactive AlkBH7 before or after annealing to the complementary strand. Subsequent treatment with DpnII cleaves repaired substrates to generate a higher mobility (lower) band. DpnII was added to both control reactions (1st and 2nd Lanes) in the dsDNA experiment.

A



B

Template	dsDNA								ssDNA						
AlkB/AlkBH7	-	-	-	-	B	7	Cl 7		-	-	-	-	B	7	Cl 7
AAG	-	-	+	+	+	+	+		-	-	+	+	+	+	+
Base	-	+	-	+	+	+	+		-	+	-	+	+	+	+

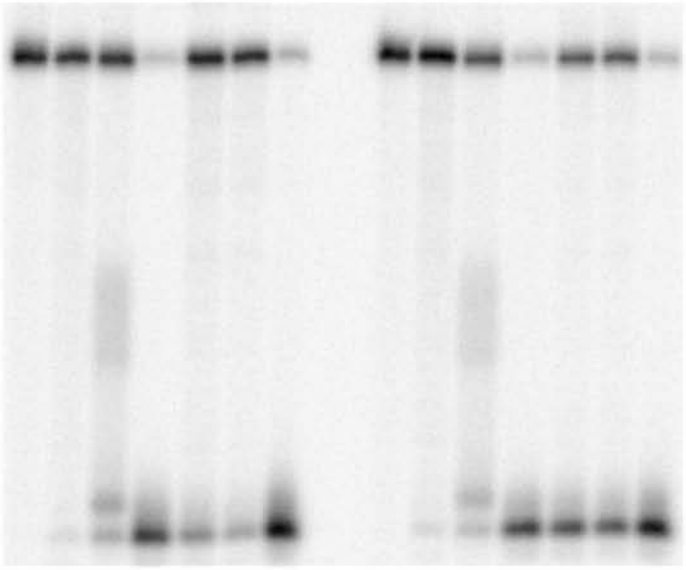


Figure 4.3 Glycosylase Sensitivity Assay (GSA) for εA removal by AlkB/ AlkBH7 on dsDNA and ssDNA Templates. A: Glycosylase sensitivity assay experimental design. 62-mer oligonucleotides containing a single εA are incubated with AlkB/AlkBH7. Subsequent processing with AAG to excise un-repaired εA bases followed by AP site cleavage with either AP endonuclease or alkali results in εA containing species being cut to 27-mer oligos. Repaired oligos are not substrates for AAG/ AP cleavage and migrate as 62-mers. Oligonucleotide substrates can be annealed prior to or following AlkB/AlkBH7 incubation to assay for activity on single-stranded or double stranded templates. B: εA repair by AlkB/ AlkBH7 as assayed by GSA. Initial incubation included AlkBH7 elution buffer, AlkB (B), AlkBH7 (7) or catalytically inactive AlkBH7 (CI 7). Repair reactions were conducted prior to (ssDNA) or after (dsDNA) annealing lesion-containing oligonucleotides to their complement. Reactions contained 200 nM enzyme and 1 nM substrate.

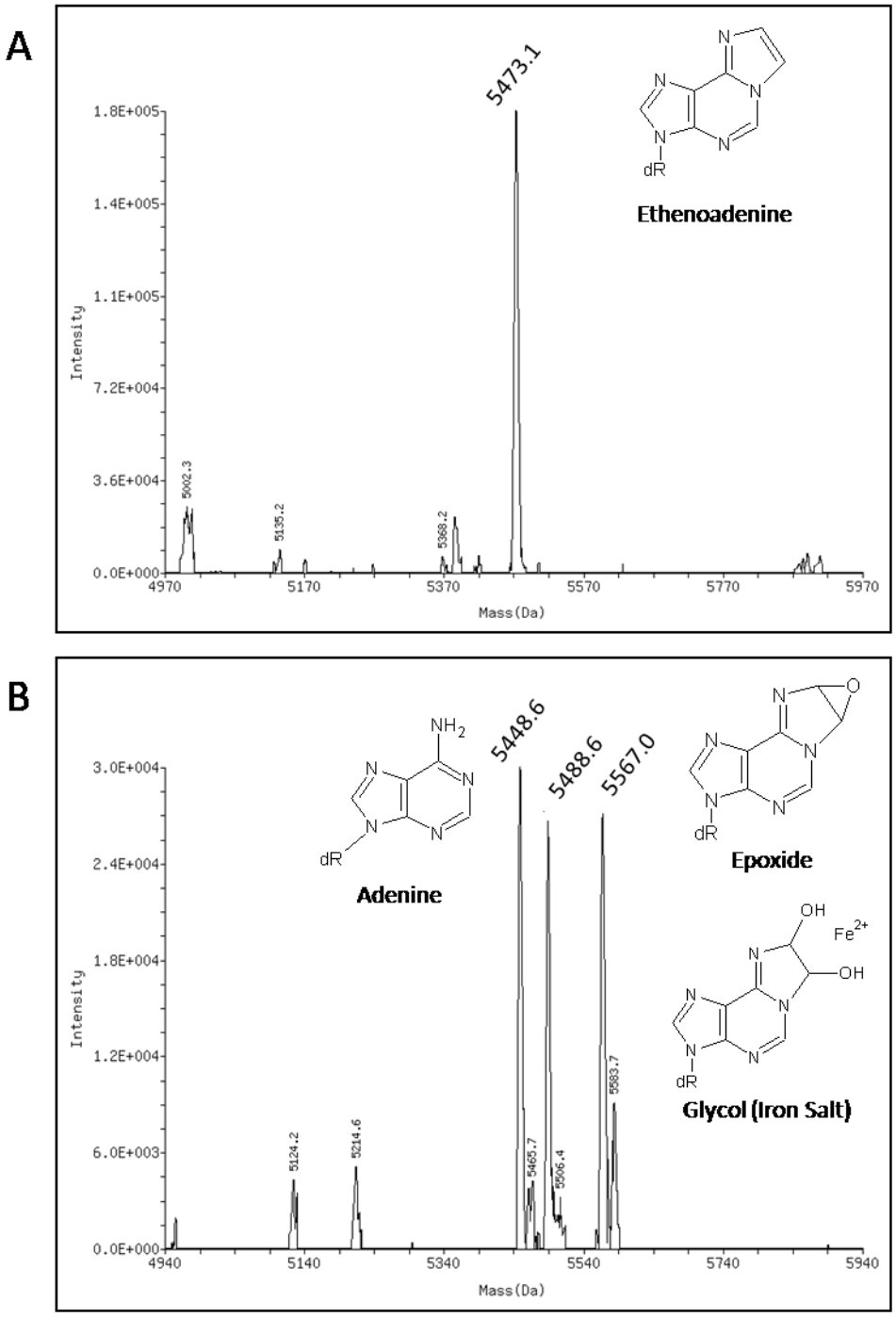


Figure 4.4: Direct removal of the etheno adduct from εA by AlkBH7. Mass spectrometry was used to characterize the molecular masses of an εA containing 18-mer oligonucleotide (A) and of the products of the incubation of that same εA containing 18-mer with AlkBH7 (B).

AlkBH7 L110Q are capable of creating AAG/ alkali insensitive sites in the substrate. In similar experiments, the extent to which AlkBH7 created glycosylase insensitive sites on a double-stranded template was greater than that on a single-stranded template. Conversion of εA containing oligomers from a 62 bp long species to a 27 bp species was nearly 95% efficient and dependent upon both the addition of AAG and alkaline hydrolysis (Figure 4.3 Panel B). The uncleaved band observed in lane 4 likely represents εA-containing oligos that did not have εA lesion removed by AAG during that reaction. A consistent result of these experiments was that AlkBH7 containing reactions showed a higher level of activity against εA lesions than AlkB.

In order to demonstrate that the glycosylase insensitivity observed in the AlkB/ AlkBH7 treated lanes was due to the direct removal of the etheno lesion from εA, mass spectrometry was used to analyze the products of the direct reversal reaction. Single-stranded 18-mer oligos containing εA were incubated with AlkB or AlkBH7 before analysis. The untreated oligo had an atomic mass of 5473.1 Da. After treatment with AlkB or AlkBH7 the major species observed was 5448.6 Da. The 24.5 Da difference can be attributed to the removal of the 2-carbon etheno lesion from the substrate (Figure 4.4). Two additional species are observed. A species with a mass of 5488.6 Da likely represents the epoxide reaction intermediate. The 5567.0 Da species is believed to represent the glycol intermediate proposed by Delaney associated with a Fe^{2+} ion which is in molar excess in the reaction.

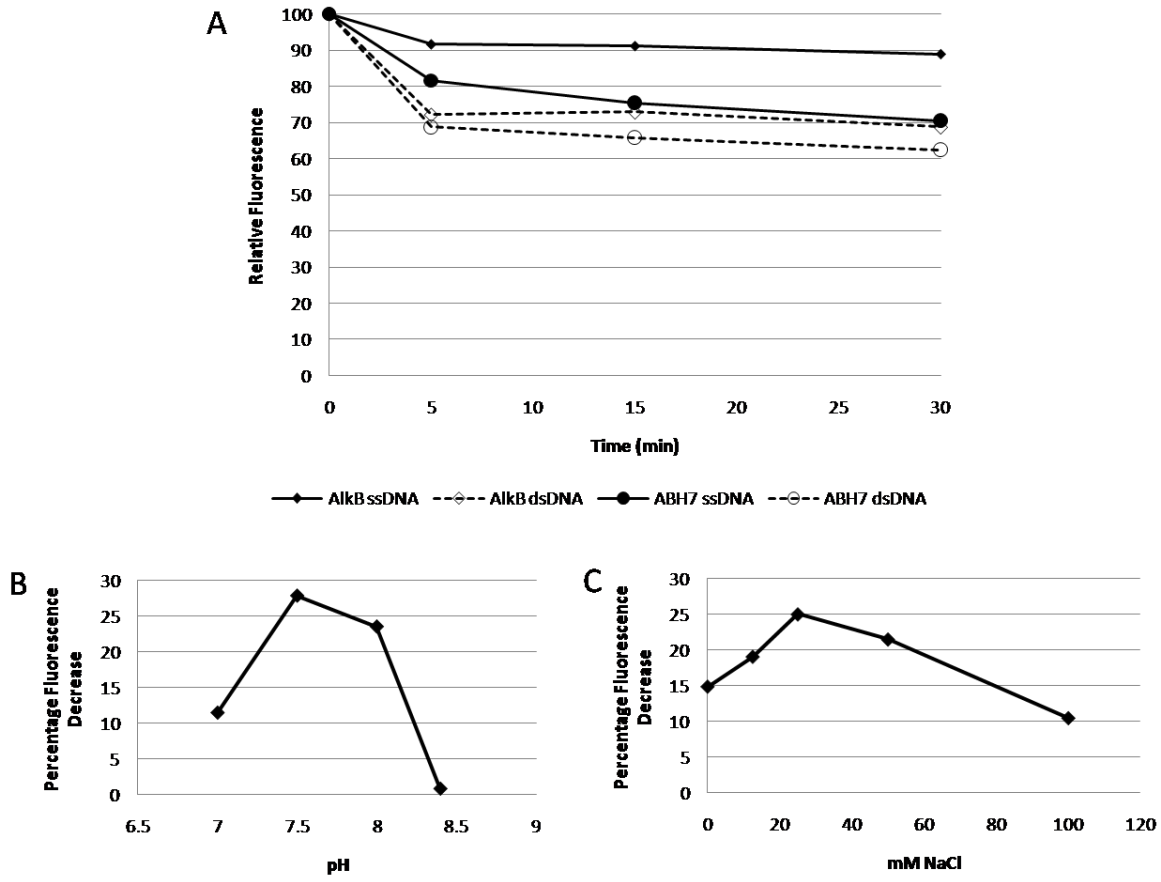


Figure 4.5: εA Fluorescence-based Assay for Direct Removal of Etheno Adduct by AlkB/ AlkBH7. A: εA fluorescence decreases during incubation with AlkB/AlkBH7. Reactions containing 228 nM single- stranded or double-stranded εA containing substrates were incubated with 135 nM AlkB/ AlkBH7 in a standard reaction buffer. εA fluorescence was measured at 406 nm after excitation at 315 nm. B: pH dependence of εA removal by AlkBH7 on dsDNA substrates. C: The effect of salt concentrations (NaCl) on εA removal by AlkBH7.

The fluorescent property of εA (Leonard, 1984) was also used to study the time dependent and substrate requirements for AlkBH7 mediated direct reversal. εA containing reactions (228 nM) were measured prior to the addition of AlkB or AlkBH7 to establish initial fluorescence readings. Addition of either AlkB or AlkBH7 (135 nM) to the reaction mixture resulted in a time dependent decrease in the εA fluorescence signal. The reaction appeared to saturate after 10-15 following a fast initial decrease in fluorescence. In similar experiments, pre-incubation of the enzyme for 10 minutes prior to the addition of the εA substrate resulted in no subsequent fluorescence decrease. This suggests that the enzyme may not be stable under reaction conditions for reasons that have not yet been determined. Despite the limited extent of these reactions, they are sufficient to show that AlkBH7 reduced εA dependent fluorescence more rapidly and to a greater extent than AlkB from dsDNA Similar results were obtained for ssDNA substrates although the overall fluorescence decrease in dsDNA substrates was 4-fold greater than those for ssDNA. This indicates that both AlkB and AlkBH7 have a preference for dsDNA vs ssDNA on εA substrates (Figure 4.5 Panel A).

Further testing was done with this technique to determine optimal pH and salt concentrations for the enzymatic reaction. There is little difference between reactions conducted on dsDNA substrates between pH 7.5 and 8.0 (Figure 4.5 Panel B). The enzyme activity was decreased at pH 7.0 and ablated at 8.4. Optimal in vitro NaCl concentration was determined to be 25 mM with inhibition of activity at 100 mM (Figure 4.3 Panel C.

Discussion

AlkBH7 contains the necessary amino acids and secondary structural elements to adopt an AlkB-like conformation and conserve the catalytic activity of the AlkB protein. These experiments were designed to test whether AlkBH7 maintained the ability to contribute to direct removal of DNA damage similar to the E. coli protein. Given that the amino terminus of the protein is sufficient to drive the protein to the mitochondria of mammalian cells, and that the amino-terminal MTS of many mitochondrial proteins is cleaved upon import to the mitochondria, a construct lacking the minimal MTS and including a C-terminal 6x his affinity tag was created and the protein expressed in E. coli to achieve high yields. A two-step purification procedure was sufficient to purify the protein to homogeneity.

The catalytic activity of AlkB as an Fe^{2+}- and α-ketoglutarate dependent dioxygenase has only recently been understood (Falnes et al., 2002; Trewick et al., 2002). Despite the well-characterized contribution of AlkB to sensitivity to S_N2 alkylating agents, the biochemical mechanism of AlkB remained elusive until these cofactor requirements were defined (Aravind, 2001). S_N2 alkylating agents include MMS which produces, among others, 1-methyladenine and 3-methylcytosine in reactions with DNA (Warwick, 1963). These base lesions are potentially mutagenic if unrepaired. By removing the methyl group from 1-meA, AlkB mitigates the potential mutagenicity directly without requiring AP endonucleases, DNA polymerases or DNA ligases.

Several human homologs of AlkB have been tested for the ability to catalyze direct removal of DNA adducts (Lee et al., 2005). AlkBH1, 2 and 3 demonstrate

significant demethylase activity while the assay of others showed no activity. Notably, when AlkBH7 was tested in this previous report, the amino terminal MTS was left intact on the purified protein; further the protein was only tested against 1-meA (Lee et al., 2005). In our study, the amino terminal MTS of AlkBH7 was removed to mimic the presumed mature mitochondrial protein. Whether this sequence contributes to regulation of enzymatic activity has not been addressed. Similar to previous reports the ability of AlkBH7 to catalyze the direct removal of the methyl group from 1-meA was poor. However, the enzyme displays significant activity against 3-meC and εA lesions in dsDNA. In comparison to AlkB, AlkBH7 removal of εA fluorescence occurs more rapidly and occurs to a greater extent, although the overall extent of the reaction is limited.

A catalytically inactive form of AlkBH7 featuring a Leu→ Gln mutation which inhibits coordination of α-KG (Sundheim et al., 2006), was included in these assays. This was a critical control as the BL21 strains used to express the protein were AlkB proficient although the high salt washing step employed during the nickel affinity chromatography step should have eliminated most contaminating proteins. This protein was purified under identical conditions to active AlkBH7 but was incapable of supporting repair. While the result is not unexpected, the implication that the inactive protein purified under identical conditions to the active form suggests that the ΔN17-AlkBH7-6his is free of contaminating AlkB activity and reinforces the conclusion that AlkBH7 is responsible for the activity observed.

Efforts have been made to optimize the assay for biochemical activity of AlkBH7 based on decreased εA fluorescence. A pH optimum of the protein was established

between pH 7.5 and 8.0. This represents a common physiological pH range for the mitochondrial matrix, the physical location of expressed protein (Abad et al., 2004; Llopis et al., 1998; Porcelli et al., 2005). When tested at pH 8.4, AlkBH7 shows no ability to remove εA in vitro. Previous descriptions of the catalytic activity of AlkB family proteins have been done in reaction conditions that lacked appreciable salt concentrations (Falnes et al., 2002; Lee et al., 2005; Trewick et al., 2002). Using rapid detection of εA fluorescence, study of the optimal NaCl concentration was conducted to optimize activity. AlkBH7 decreases overall εA fluorescence at 25 mM NaCl; however the sensitivity to salt was not as great as to pH. While physiological salt concentrations seem to limit the extent of the enzyme reaction, the relationship between optimal in vitro assay conditions for purified protein and in vivo activity of expressed protein can be modulated by many factors including the nature of the salts, abundance of cofactors, or in vivo protein modifications and interactions.

Previous reports of enzymatic activity of AlkB family members have not reported definitive kinetic data in reactions with excess substrate. Enzyme assays with labeled oligonucleotide substrates are often conducted under conditions of enzyme excess, and most preparations appear to saturate before complete repair of the targeted DNA lesion. This implies that optimal assay conditions have yet to be established. Attempts were made to improve the assay conditions in these experiments but only limited results were achieved. Potentially, AlkB family members function not alone on DNA but in concert with other proteins to catalyze direct repair of DNA damage. These proteins could affect the binding of the protein to DNA, the ability to exchange α-KG, or the accessibility of molecular oxygen. Alternatively, in vivo, AlkB family proteins could be targets for post

translational modification that modulates activity in response to DNA damage or other stimuli which are not reflected in proteins purified from prokaryotes.

A substantial difference in activity between the glycosylase sensitivity and fluorescence assays is observed. In the GSA, the relative concentration of the enzyme to DNA was much higher (estimated to be 200 nM protein to 1 nM substrate) than in the fluorescence experiments in which the substrate concentration was closer to the enzyme concentration (228 nM εA to 135 nM protein), the εA fluorescence decreased by 25- 30% at maximum indicating that the reaction was incomplete. Further after 5 minutes in the assay conditions, the ability of AlkBH7 to remove fluorescent signature of εA was significantly compromised. Taken together, this data suggests that either the preparation of AlkBH7 is composed of active and inactive fractions, which are indistinguishable by MW analysis or elution profile from the MonoS column, or that the conditions of the assays foster an undescribed instability in the enzyme.

One distinct possibility that both the fluorescence and glycosylase sensitivity assays leave open is that AlkBH7 does not completely remove the εA lesions from the DNA, rather the εA substrate could be converted to a ring-open N^6 acetaldehydic isomer which would be non-fluorescent and a poor substrate for AAG (Frick et al., 2007). This could potentially limit the mutagenicity of εA upon replication, by forming normal Watson-Crick base pairs with thymine bases. MS analysis of the products of reaction of εA with AlkBH7 shows that the predominant product has a weight 24.5 Daltons less than the starting material. This decrease in weight corresponds to the weight of 2 carbons atoms, the constituents of the etheno adduct. A product of the reaction 15.5 Da larger than the starting material is observed in the mass spectra. Two potential interpretations

of this species can be offered. One interpretation is that the reaction proceeds through an epoxide intermediate proceeding to a glycol before release of the etheno adduct as glyoxal. This reaction mechanism is identical to that proposed for the removal of εA by AlkB (Delaney et al., 2005). Alternatively, hydroxylation of the N^1 position, leaving a +16 mass on the acetaldehydic intermediate, and restoring the base pairing capacity of adenine can explain this species as observed in the alleviation of 1, N^6- ethanoadenine by AlkB (Frick et al., 2007). A second major species occurs at 5567.0 Da. Explanation of this species is more difficult but may represent the glycol intermediate with an associated Fe^{2+} ion or an artifact of processing containing multiple K+ adducts. The abundance of the reaction intermediates observed may be due to the substrate: enzyme ratio. The molar concentration of εA substrate was calculated to be 1.5 μM while the AlkBH7 concentration was 80 nM.

MGMT is a direct reversal enzyme that transfers aberrant methylations on the O^6 position of guanine to itself thus inactivating the enzyme (Lindahl, 1982). In experiments measuring the decrease of εA fluorescence over time, AlkBH7 shows a similar kinetic behavior to MGMT acting on O^6-methylguanine. The possibility that the dialdehyde glyoxal produced by the removal of the etheno lesion reacts with and inhibits the enzyme may contribute to the limited activity observed. The evolution of formaldehyde from reactions containing methylations and glyoxal from repair of etheno lesions has been observed for AlkB, but the means to assay for the generation of these products by AlkBH7 were unavailable. Thus, failure of AlkBH7 to release these products cannot be ruled out.

Only DNA substrates were tested in these experiments. RNA substrates have been considered and in some cases studied with other AlkB family proteins, so the possibility that AlkBH7 contributes to RNA repair or methylation cannot be discounted.

Taken as a whole, the biochemical data demonstrates that AlkBH7, like AlkB, is capable of catalyzing the direct removal of the methyl group from 3-meC and the etheno adduct from εA. Both enzymes prefer dsDNA templates. The product of the reaction is likely a normal cytosine or adenine base, respectively. Given that previous attempts to describe the catalytic activity of AlkBH7 employed a full length protein, it is possible that the presence of the MTS may inhibit the protein until mitochondrial import has been achieved.

Chapter 5: Repair of mtDNA by AlkBH7

Introduction

As has been discussed, the mitochondrial genome exists in an environment rich in ROS which can act directly or indirectly to damage the mitochondrial genome. Endogenous levels of 8-oxo-guanine in mtDNA reflect this type of environment (de la Asuncion et al., 1996) (Hegler et al., 1993; Trapp et al., 2007). MtDNA is also subject to higher levels of lipid peroxidation than nuclear DNA as evidenced by M1G lesions (Jeong et al., 2005). While the mtDNA is dispensable in cultured cells (King, 1989), mutation, depletion or deletion of mtDNA results in multiple and varied pathologies.

AlkBH7 is capable of contributing to repair of 3-meC and εA lesions in both single-stranded and double-stranded DNA in vitro. However, the contribution of AlkBH7 to in vivo repair is unknown. MMS- induced lesions, which can include 1-meA and 3-meC, in mtDNA are repaired effectively (Pirsel, 1993; LeDoux et al., 1999; LeDoux et al., 1993). Evidence for repair of oxidative damage suggests that BER contributes to mtDNA maintenance (Grishko et al., 2005; Karahalil et al., 2003). Mitochondrial BER enzymes associate with the inner mitochondrial membrane and are independent of mtDNA presence (Stuart et al., 2004; Stuart et al., 2005). Despite the description of AlkBH1 as a mitochondrial protein, no evidence exists that direct repair can contribute to repair of mtDNA.

Chloroacetaldehyde (CAA) is a bifunctional aldehyde produced during the metabolism of vinyl chloride (Green, 1978). The reaction of CAA with DNA can produce εA, εC, $1N^2$-ethenoguanine and N^2-3 ethenoguanine bases in vitro and in vivo

(Green, 1978; Guengerich, 1992). The presence of these lesions is mutagenic in E.coli and the mutational spectrum is dependent on the ADA response (Mroczkowska et al., 1993). As part of the adaptive response, both AlkA (ANPG, MPG, AAG) and AlkB contribute to repair of these lesions.

Experiments were undertaken to investigate whether AlkBH7 could contribute to mtDNA repair in vivo. Initial experiments asked whether AlkBH7 could complement AlkB deficiency in E. coli using a plasmid reactivation assay. To test whether expression of AlkBH7, could contribute to cellular viability after exposure to DNA damaging agents, clonal cell lines expressing AlkBH7 in HeLa cells were created and assayed for viability following exposure. Finally, mtDNA from cells exposed to CAA was analyzed for the presence of AAG sensitive sites in an assay similar to the GSA assay used for etheno removal from εA.

Materials and Methods

AlkB Complementation

The alkB deleted strain of E. coli K-12 (JW2200) was obtained from the E. Coli Genetic Stock Center at Yale University. AlkB-6xHis and AlkBH7-6XHis cassettes were moved from pET22B expression vectors into the TAC-promoter expression plasmid pMMB207. E. coli JW2200 cells were transformed with the plasmid and transformants selected on chloramphenicol-containing media. E. coli cultures containing the AlkB-his and AlkBH7-his TAC expression plasmids, respectively, were grown in the presence of chloramphenicol and 1 mM IPTG to an OD of 0.4 at which time they were made competent by successive washes in 80 mM $CaCl_2$/ 20 mM $MgCl_2$ then 100 mM $CaCl_2$. DMSO was added as cryoprotectant and aliquots of the competent cells stored at -80 °C.

Treatment of DNA with chloroacetaldehyde (CAA) was accomplished as follows: 2 μg of either single-stranded pBS- or double-stranded pBS+ were treated with 10 mM CAA in 25 mM sodium cacodylate buffer, pH 7.25, at 37 °C for various time intervals. At indicated time points, the reaction was stopped by addition of 3 volumes of 0.3 M sodium acetate, pH 4.5, and 20 μg glycogen. The DNA was ethanol precipitated. Following resuspension of the DNA in 1xTE, the concentration of the DNA was determined. For transformation, 20 ng of single-stranded pBS- or 2 ng of double-stranded pBS+ was mixed with 2 ng of pET28a and transformed into calcium competent cells. The Kan^R pET28a vector provided an internal control for transformation efficiency. After recovery, equivalent volumes of the culture were plated in triplicate on LB-Amp and LB-Kan plates (for JW2200) or LB-Cam-Amp and LB-Cam-Kan plates (AlkB-6xhis and AlkBH7-6xhis strains.) Plates were incubated overnight at 37 °C. The number of colonies on each plate was counted manually. To normalize for transformation efficiency and minor differences in the OD when competent cells were made, the total number of ampicillin resistant colonies was divided by the number of kanamycin resistant colonies for each condition tested. The Amp^R/Kan^R colony ratio from cultures transformed with untreated plasmids was considered 100%. Recovery ratios reported indicate the ratio of Amp^R/Kan^R colony count ratios normalized to this value.

Cell Culture and Creation of AlkBH7 Expressing Clonal Cell Lines

HeLa cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 5% (v/v) calf serum, 5% (v/v) fetal bovine serum, penicillin and streptomycin. Cells were passaged at a 1:10 ratio every 3-4 days as necessary. To create AlkBH7 expressing clonal lines, the full length AlkBH7 expression construct used for immunofluoroescence

studies was transfected into HeLa cells using Fugene 6. Transfected cells were selected using Zeocin at 400 μg/ml. AlkBH7 expression was confirmed by reverse transcription PCR using AlkBH7 specific primers: 5'-CACTGGGACGCGGCCATCCA-3' and 5'-ATCTCATGGGAGAAGTCAT-3'.

Cell Treatment and Viability Assays

To assay for viability after CAA or H_2O_2 treatment, a 96 well format was used. 2500 cells per well were plated and allowed to adhere overnight. After 16 hours, the media was removed and replaced with media containing from 0 to 30 μM CAA or 0 to 1.5 mM H_2O_2. All chemicals were diluted from stock into complete media immediately before use. Exposure to CAA continued for 2 hours at which time the medium was removed, the cells washed 2-3 times with PBS and fresh culture medium applied. H_2O_2 containing media was not replaced. Plates were allowed to recover for 2-4 days and viability assayed using Aqueous One Reagent according to the manufacturer's instructions.

Hirt Extraction of Low Molecular Weight DNA

10 cm plates of cells were treated with 15 μM CAA for 2 hours. Plates were washed 2-3 times with PBS and fresh media applied to cells for recovery. Hirt extraction was used to isolate low molecular weight DNA (Hirt, 1967). After washing the cells, 1ml of 0.6% SDS, 10 mM EDTA lysis buffer was applied to each plate and incubated at room temperature for 10-15 minutes. The viscous lysate was collected by scraping the material into a 50 ml conical tube and pooling similarly treated plates for each sample. 5 M NaCl was added to bring the final concentration to 1 M NaCl and the tubes were stored at 4° C overnight. Insoluble material was pelleted by centrifugation at 17,000 xg for 30 minutes

and the supernatants transferred to fresh tubes. The samples were phenol: chloroform extracted twice then ether extracted, each time transferring the aqueous phase to a fresh tube. Residual ether was removed by incubating the samples at 65° C for 20-30 minutes. 2.5 volumes of 100% ethanol was added and the tubes stored at -20° C for at least 1 hour. Ethanol insoluble material was pelleted by centrifugation at 15,000 xg for 15 minutes at 4° C. Pellets were washed with 70% ethanol then dried. The pellet was resuspended in 250 μl TE.

DNA was quantified by fluorimetric analysis using PicoGreen according to the manufacturer's protocol. To determine the relative abundance of mtDNA in the sample, 1 μg of total material was digested with HindIII in the presence of RNaseA and the samples analyzed on 0.8% agarose TBE gels. Gels were stained with ethidium bromide to visualize DNA and compare the abundance of mtDNA signal in the samples.

Contribution of AlkBH7 to MtDNA Maintenance

To evaluate the contribution of AlkBH7 to mtDNA maintenance, low molecular weight DNA was isolated from HeLa and AlkBH7 expressing clonal lines prior to, immediately after or after a 4 hour recovery from exposure to 15 μM CAA for 2 hours. After quantification, 1 μg of each sample was digested with BamHI in the presence of RNaseA. Sequential processing with AAG and APE was done each for 60 minutes in appropriate buffers. Following the enzymatic processing, the samples were ethanol precipitated and resuspended in 20 μl TE. 5 μl of the volume was applied to a 0.8% (w/v) TBE agarose gel. The remainder of each the sample was heated to 90° C for 5 minutes then snap chilled and briefly centrifuged. 3 μl of 6X Alkaline Loading Buffer (300 mM NaOH, 10 mM EDTA, 18% (w/v) Ficoll 400, 0.25% (w/v) Bromocresol Green,

0.25% (w/v) Xylene Cyanol) was added and the samples were loaded onto a 1% alkaline agarose gel (cast and run in 23 mM NaOH, 1 mM EDTA.) Following electrophoresis, gels were stained with ethidium bromide and imaged then nucleic acids were transferred to Hybond N+ by alkaline transfer (Sambrook et al., 1989). Blots were cross-linked first by UV irradiation then by baking for 2 hours at 80° C in a vacuum oven.

CsCl purified mtDNA was used to generate a ^{32}P-labeled probe by random priming and insertion of α-^{32}P-dCTP by the Klenow fragment of Pol I. Unincorporated label was removed by ethanol precipitation and the probe was resuspended in TE.

Blots were pre-hybridized for 1-2 hours at 37° C in Formamide Hybridization Buffer (Sambrook et al., 1989). The probe was denatured by heating to 90° C for 5 minutes then added to the prehybridization solution. Hybridization was conducted at 37° C for 18-20 hours. Blots were washed twice with 2X SSPE, 0.1% SDS for 15 minutes at room temperature then twice with pre-warmed 0.2X SSPE, 0.1% SDS at 42° C for 15 minutes. A final wash with 2X SSPE for 5 minutes was conducted at room temperature. The blots were visualized by exposing a phosphor-storage screen and imaged with a Storm Phosphorimager.

Results

In order to determine whether AlkBH7 could complement AlkB deficiency in E. coli, an experimental system was devised that relied on the reactivation of CAA-treated pBlueScript DNA by AlkB/AlkBH7 to confer resistance to ampicillin- containing growth media. Chloramphenicol-selectable expression plasmids containing either the coding sequence for AlkB or ΔN17AlkBH7 under the control of a TAC promoter were transfected into E.coli. Chemically competent E.coli were prepared from cultures grown

in the presence of chloramphenicol to allow selection of only cells containing the expression plasmid and IPTG to allow for expression of either AlkB or AlkBH7. Single-stranded or double-stranded pBlueScript was treated with CAA in vitro, precipitated, quantified and used to transform chemically competent E. coli. As a control for transfection efficiency, Kan^R pET22b+ was co-transformed. Transformed cultures were plated onto either ampicillin-containing or kanamycin-containing selective media in triplicate. After growth overnight at 37 °C, the number of colonies on each plate were counted and reported as the number of Amp^R/Kan^R colonies per condition. Results are shown in Figure 5.1. Panel A shows that expression of AlkB but not AlkBH7 increases the percentage of Amp^R/Kan^R colonies in CAA treated single-stranded pBlueScript. Panel B shows that with CAA treated double-stranded pBlueScript, the Amp^R/Kan^R ratio increases as a consequence of either AlkB or AlkBH7 expression. As expected, expression of AlkB in E. coli results in a greater recovery from CAA treatment than does AlkBH7. This may be due to poor expression or solubility of AlkBH7 in the E. coli strain used. Nonetheless, in otherwise isogenic strains of E. coli, AlkBH7 is capable of enhancing the ability to resolve etheno- lesions in dsDNA by 30% over the wild-type.

To study the contribution of AlkBH7 to repair of the mitochondrial genome, stable cell lines containing plasmids allowing expression of full length AlkBH7-myc-his were isolated from HeLa cells using resistance to Zeocin encoded by the plasmid. Clonal

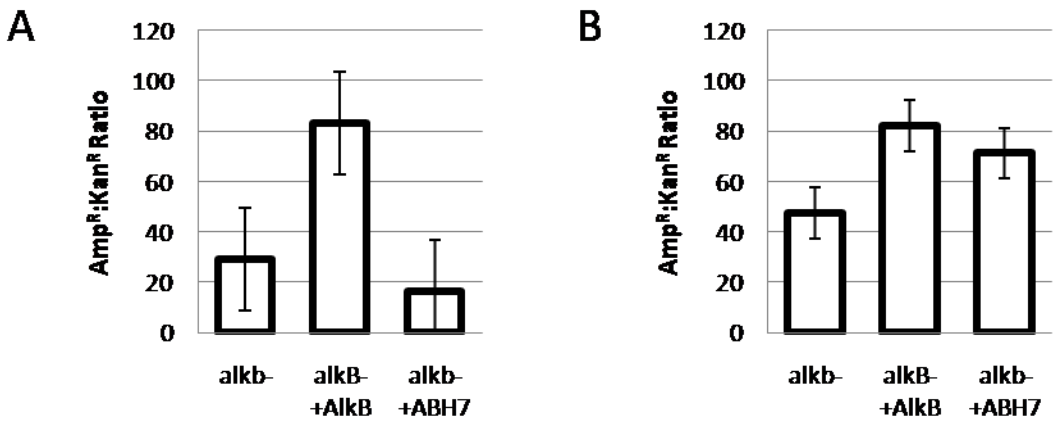


Figure 5.1 Complementation of AlkB deficiency by AlkBH7 in E. coli. Chemically competent E. coli expressing AlkB or AlkBH7 were co-transformed with CAA-treated single-stranded pBlueScript (A) or double-stranded pBlueScript (B) and pET22B^{+}. Transformations were allowed to recover and plated on either ampicillin or kanamycin containing plates in triplicate. After growth overnight at 37° C, the number of colonies on each plate was counted and the ratio of ampicillin to kanamycin resistant colonies was calculated. The number of Amp^{R} colonies for each condition was normalized against the number of Kan^{R} colonies for that same condition to account for differences in cell density at the time the cells were made competent.

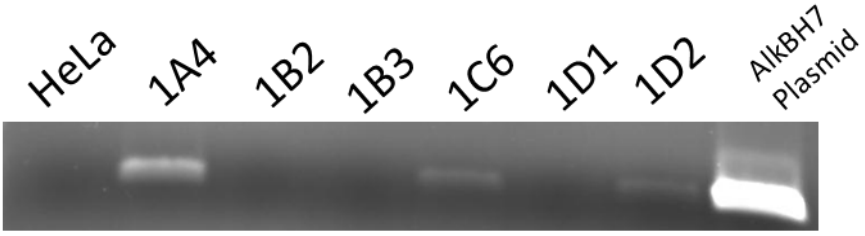


Figure 5.2: Identification of AlkBH7 Expressing Clonal Cell Lines by RT-PCR. 1 μg of total RNA was used to generate first strand cDNA using oligo-dT to prime reverse transcription. PCR using AlkBH7 specific primers was then conducted. AlkBH7 containing plasmid DNA was used as a positive control for the amplicon.

cell lines were selected with 400 μg/ ml Zeocin and assayed for expression of AlkBH7 by reverse-transcription PCR (Figure 5.2.) Based on these results, 3 lines were selected, 1A4, 1C6 and 1D2.

As etheno lesions can be generated by exposure to CAA as well as a result of lipid peroxidation, cellular viability was measured in these cell lines following CAA exposure. CellTiter 96® AQueous One Solution Reagent (Promega) was used to measure mitochondrial succinate dehydrogenase (mitochondrial complex II) activity as a surrogate for viability. CAA exposures were brief, limited to 2 hours after which cells were allowed to recover before the MTS assay was conducted. Initial experiments indicated that the parental line (HeLa) as well as each clonal line had an LC50 of 15 μM for a 2-hour exposure followed by 48 hours of recovery. 48 hours after a 2 hour exposure to CAA, both HeLa and AlkBH7 expressing clonal lines (average) demonstrated LC50 values of 15 μM. With longer recovery periods, AlkBH7 expression confers a greater tolerance to CAA exposure with LC50 concentrations of 15 μM for HeLa cells after 72 and 96 hours, but 18 μM as an average for the AlkBH7 expressing clonal lines (Figure 5.3). Exposure to H_2O_2 was tested similarly, however the media was not replaced after 2 hours. 48 hours after H_2O_2 exposure, there is no difference in the succinate dehydrogenase activity of the parental line versus the AlkBH7 expressing clonal lines (Figure 5.4).

While viability measures the overall fitness of cells in culture following exposure to a cytotoxic agent, the contribution of mtDNA maintenance to overall cellular viability is uncertain. To more directly assess the impact of AlkBH7 expression on the mtDNA, I

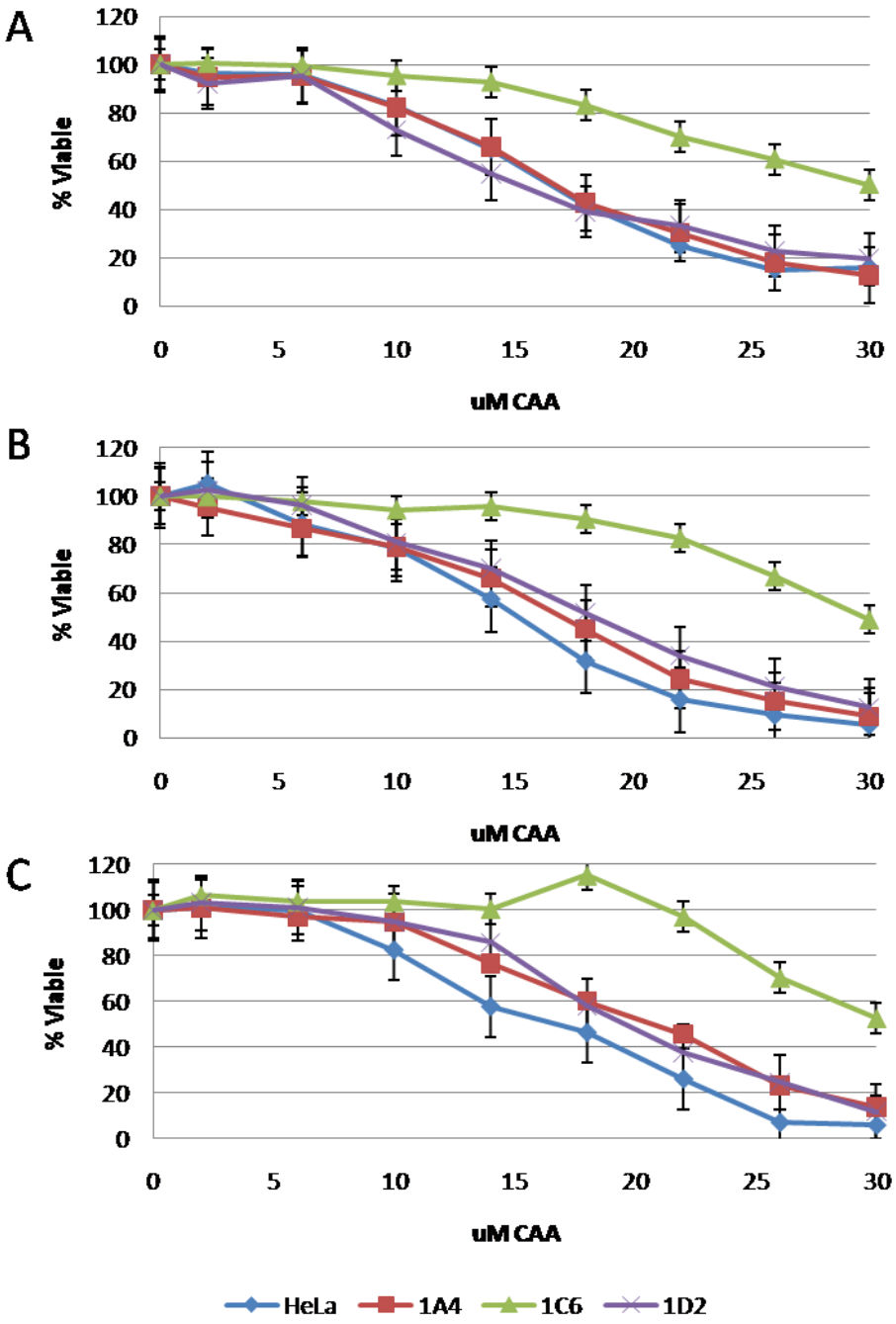


Figure 5.3 Expression of AlkBH7 increases viability following CAA exposure. HeLa or AlkBH7 expressing clonal cell lines were plated in 96 well plates and allowed to seat overnight. CAA was added to the media bringing final concentrations to between 0 and 30 μM for 2 hours. Cells were washed twice with PBS and fresh growth media added. Viability as measured by MTS reduction by succinate dehydrogenase was measured 48 (A), 72 (B) and 96 (C) hours after growth media was added.

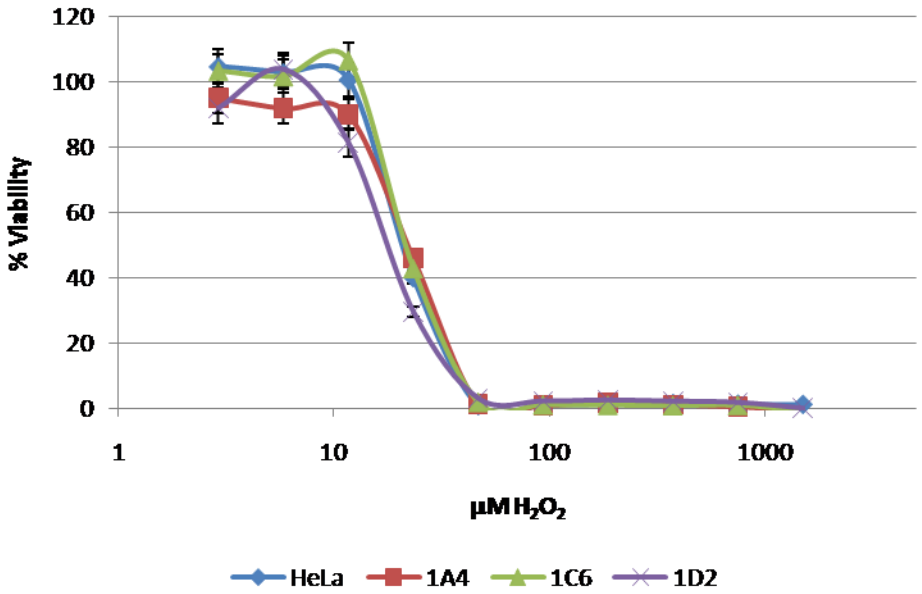


Figure 5.4 Expression of AlkBH7 does not affect viability following H_2O_2 exposure. HeLa of AlkBH7 expressing clonal cell lines were plated in 96 well plates and allowed to seat overnight. H_2O_2 was added to final concentrations between 0 and 1.5 mM. Viability as measured by MTS reduction by succinate dehydrogenase was measured 48 hours after H_2O_2 exposure.

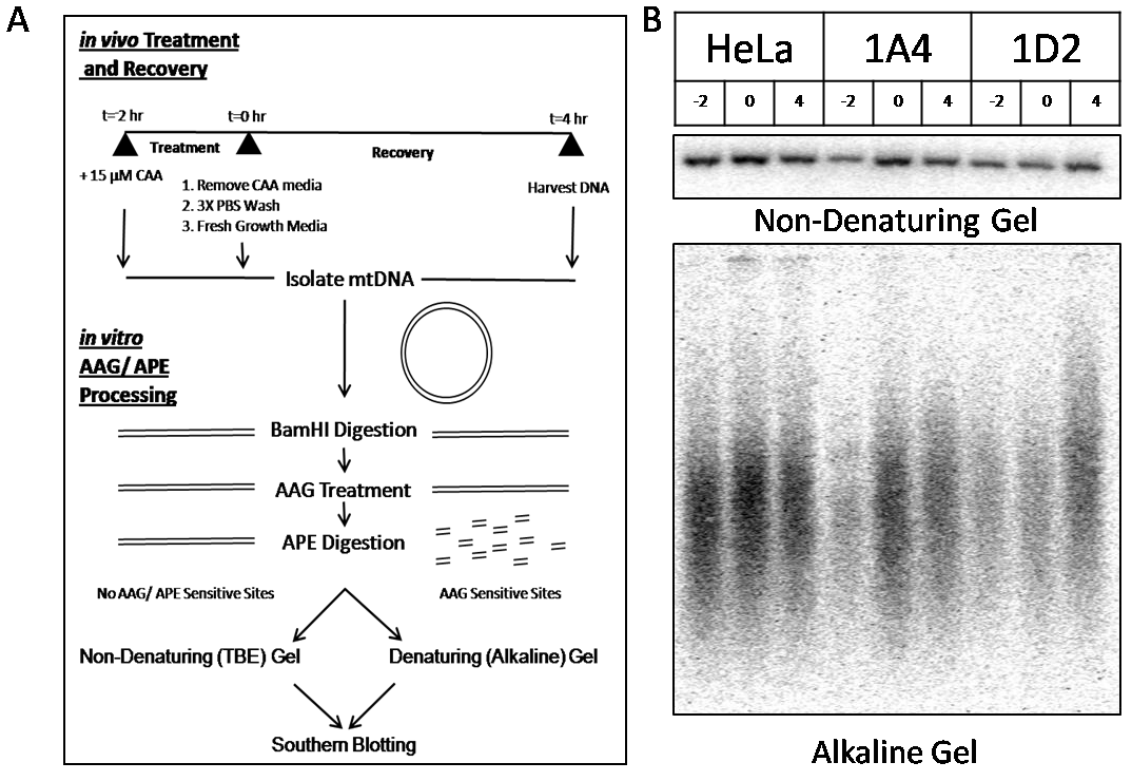


Figure 5.5 AlkBH7 contributes to repair of mtDNA in vivo. A. Schematic representation of the assay employed to analyze repair of mtDNA by AlkBH7. B. Low molecular weight DNA isolated from HeLa and AlkBH7 expressing clonal lines 1A4 and 1D2 was treated sequentially with BamHI, AAG and APE before analysis for mtDNA on non-denaturing (TBE) or denaturing agarose gels. MtDNA was detected by Southern hybridization using a probe against HeLa mtDNA.

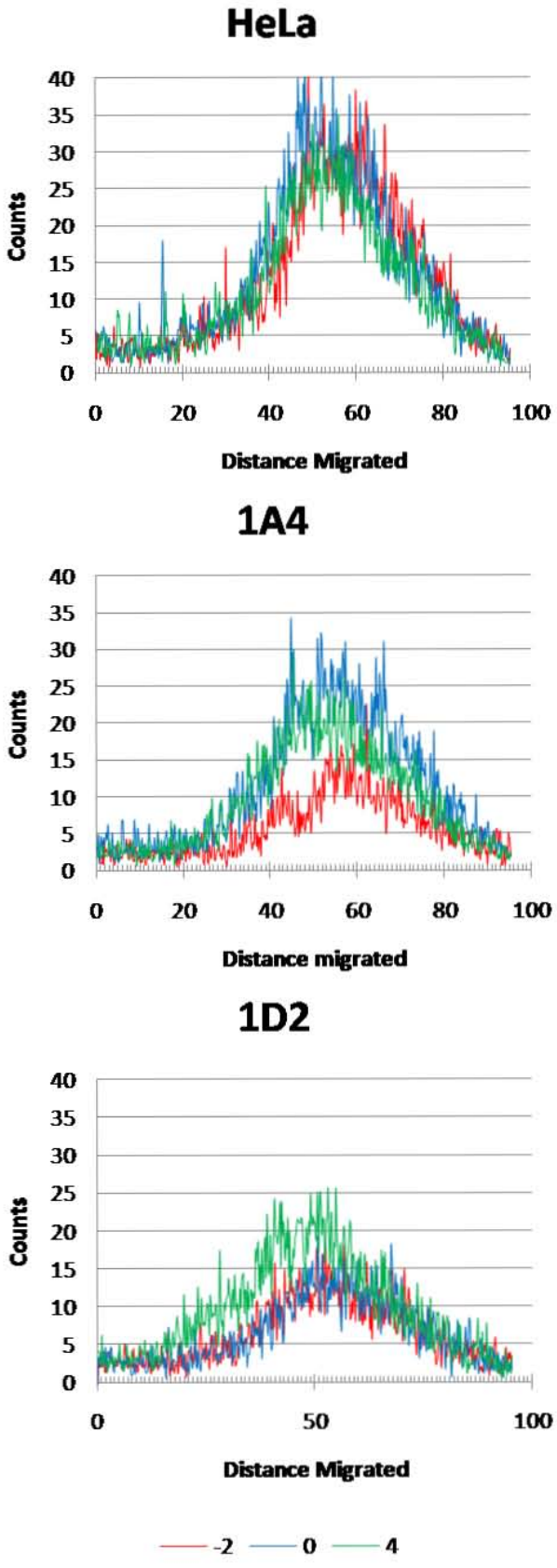

HeLa
Counts
Distance Migrated
1A4
Counts
Distance migrated
1D2
Counts
Distance Migrated
-2
0
4

Figure 5.6 Quantification of Alkaline Fragmentation Pattern of mtDNA from Figure 5.5. The fluorescence signal intensity over the length of the blot, starting from the location of the well to the bottom of the blot along a line running down the center of each lane, was determined using ImageQuant. The results were graphed as a property of the signal intensity versus the distance migrated. Red lines represent the fragmentation pattern of mtDNA prior to CAA exposure, blue lines represent the fragmentation pattern immediately following exposure and green lines represent the fragmentation pattern observed after 4 hours of recovery.

analyzed incidence of AAG-cleavage sites in the mtDNA of HeLa and AlkBH7 clonal lines exposed to CAA and allowed to recover. This is an endonuclease-susceptible site assay similar to the GSA assay used to study the contribution of AlkBH7 to repair of εA lesions in vitro (Figure 5.5 Panel A). MtDNA was extracted from cells prior to, immediately following, or after a period of recovery from exposure to 10 μM CAA. Following processing with AAG and APE, the mtDNA was separated on either a non-denaturing TBE gel or a denaturing alkaline gel before being transferred to a membrane for detection using an mtDNA specific probe. Results are shown in Figure 5.5 Panel B. Under non-denaturing conditions, mtDNA appears as a discrete species despite endonuclease processing. Denatured DNA run under alkaline conditions, mtDNA appears fragmented by the AAG/APE processing into a smear of lower molecular weight species. Although this assay is complicated by the known incidence of alkali-susceptible sites in mtDNA, I reasoned that the additional burden of AAG-susceptible sites induced by CAA-treatment could be detected by a reduced chain length of mtDNA on alkaline gels. The fragmentation pattern observed suggests that CAA has little effect on the fragmentation pattern of the mtDNA (Figure 5.5 Panels B and C) which could be difficult to detect given the abundance of alkaline labile sites in the mtDNA (Grossman et al., 1973; Lonsdale and Jones, 1978; Wong-Staal et al., 1973). 4 hours of recovery following CAA exposure allows the fragmentation pattern of mtDNA from AlkBH7 expressing lines 1A4 and 1D2 to shift towards lower mobility species indicative of fewer alkaline labile/ AAG/APE sensitive sites while the HeLa parental strain shows no change (Figure 5.5 Panel B and C.)

Discussion

This chapter demonstrates that AlkBH7 is capable of contributing to repair of mtDNA in vivo. The ability to complement AlkB deficiency in E. coli demonstrates that AlkBH7 is active as a direct reversal enzyme. AlkBH7 expression correlates with increased tolerance towards CAA exposure yet little resistance to H_2O_2. Although etheno lesions are not the sole substrates for AAG, the abundance of alkaline labile/ AAG/APE sensitive sites decreases more rapidly following CAA exposure in AlkBH7 expressing cells.

E. coli complementation has been used to study the ability of AlkBH1, 2 and 3 to contribute to DNA repair of methylated DNA (Duncan et al., 2002). Reactivation of damaged phage DNA has been used to characterize the SOS and ADA responses in E. coli (Ajioka et al., 2006; Bertani, 1953; Yamamoto et al., 1978). Although distinct from a phage reactivation assay, the plasmid reactivation assay used in these experiments is functionally similar to the phage activation assay. Methylation of nucleobases by MMS or MNNG produces DNA lesions that can be mutagenic if unrepaired. Similarly in vitro treatment of DNA with CAA forms etheno-lesions (Lee, 1973; Oesch, 1982) that can result in mutation if unrepaired (Borys et al., 1994; Young Kim et al., 2007). However CAA itself does not induce the adaptive response in E. coli (Mroczkowska et al., 1993). In this assay, the ability of ethenolated (CAA treated) plasmid DNA to confer resistance to selective agents is dependent on DNA repair processes of the E. coli in question. The cells used in this study were not stimulated to induce expression of adaptive response genes, thus, theoretically, limiting the background levels of repair. Induction of adaptive

response elements subsequent to transformation with ethenolated plasmid cannot be excluded as having contributed to the viability observed.

If repaired by AlkA utilizing BER, ethenolated ssDNA would be fragmented resulting in an overall loss of the ability to grow in the selective environment. Reversal of the etheno-adducts mediated by a direct reversal process would result in intact ssDNA molecules capable of supporting growth under selective pressure. As shown, expression of AlkB but not AlkBH7 in E. coli contributes to enhanced viability of cells transformed with methylated ssDNA. Repair of etheno adducts in dsDNA can occur via BER or other processes accounting for the higher background of viable E. coli in this experiment. Expression of AlkB or AlkBH7 enhances the number of viable colonies growing under selective pressure over and above this background. In light of biochemical evidence supporting higher AlkBH7 activity on dsDNA templates, the observed substrate-dependent complementation of AlkBH7 expression in E. coli is expected and validates the data.

The overall contribution of AlkBH7 to viability under selective pressure is not as great as expression of the E. coli AlkB protein. In preparing protein for biochemical studies, it was found that expression of AlkBH7 was optimal in E. coli engineered for heterologous protein expression and at reduced temperatures. The E. coli strain used to study AlkBH7-dependent complementation did not express limiting tRNAs and expression was conducted at room temperature thus the overall level of active AlkBH7 expressed was likely lower than that of the AlkB protein expressed from a similar promoter.

A slightly enhanced viability following CAA exposure was observed in mammalian cells expressing AlkBH7. Over-expression of mtDNA repair components can contribute to enhanced viability as measured by MTS reduction when challenged by oxidative stress (Dobson et al., 2000). The ability of AlkBH7 to contribute to repair of εA lesions in vitro led to experiments in which the viability of cells expressing AlkBH7 was studied following CAA exposure. The minor yet reproducible increase in LC50 values following CAA exposure observed in AlkBH7 expressing cell lines indicates that the effect is not solely dependent on mitochondrial BER, but can be a consequence of overall mtDNA maintenance. The degree of resistance to CAA exposure of individual lines was independent of AlkBH7 mRNA expression levels. As CAA is a non-specific bifunctional aldehyde, reaction with other cellular components cannot be excluded. However, in vitro biochemistry suggests that AlkBH7 can contribute to εA repair, thus the DNA lesions resulting from CAA exposure and the resolution of these lesions by AlkBH7 are the most likely cause of this enhanced viability. An artifact of in vivo experiments on tissue culture cells is the low dependence on oxidative phosphorylation as a source for ATP generation. When grown in high glucose containing media, substrate level phosphorylation is sufficient to meet many of the energy needs of the cell thus limiting the dependence on mtDNA-encoded proteins to support oxidative phosphorylation. Hence, damage to, and repair of, the mtDNA is not critical to support continued cellular viability. This may explain the modest tolerance for CAA observed in AlkBH7 expressing cell lines.

Both CAA and H_2O_2 act indiscriminately on cellular components. Modifications of nucleic acids, proteins, carbohydrates and lipids by these chemicals can contribute to

their toxicity. The small but reproducible increase in viability observed in AlkBH7 expressing cell lines is not unexpected. In tissue culture, mammalian cells can be depleted of mtDNA when supplemented with uridine and pyruvate (King and Attardi, 1989) indicating that damage to the mtDNA can then be tolerated under appropriate conditions. The degree of resistance to CAA reflects this, although longer recovery times may allow for cells with enhanced competence in maintenance of mtDNA to display an overall advantage against less competent cells in long term survival. Just as expression of an exonuclease deficient pol γ in mice leads to accelerated aging and increased levels of mtDNA heterozygousity (Trifunovic et al., 2004), expression of mtDNA maintenance factors such as AlkBH7 may contribute to longevity and delayed aging.

Repair of the mitochondrial genome under acute or chronic stress has been attributed to BER (Akbari et al., 2008; Chen et al., 2003; Dobson et al., 2000; Driggers et al., 1993; Harrison et al., 2005; Ho et al., 2007; Liu et al., 2008; Pirsel, 1993; Szczesny et al., 2008). Canonical BER utilizes lesion-specific DNA glycosylases which recognize damaged nucleobases and excise them from the DNA strand generating an AP site. AP sites are resolved by the action of class II AP endonucleases that nick the DNA backbone presenting a free 3' hydroxyl. A DNA polymerase catalyzes the insertion of a correct nucleotide using the free 3' hydroxyl as a primer. Nucleotide insertion can occur over a single base (Short Patch BER) or multiple bases (Long Patch BER.) Mitochondrial enzymes are capable of supporting many of these processes (Akbari et al., 2008; Chen et al., 2000; de Souza-Pinto et al., 2001a; Liu et al., 2008; Pinz, 1998; Stuart et al., 2004; Szczesny et al., 2008) with the presence of glycosylases in the mitochondria being the limiting factor. Although AAG has the ability to excise a broad set of DNA lesions (Lee

et al., 2009), the presence of the enzyme or similar activity has not been described in mitochondria or mitochondrial extracts. The contribution of AlkBH7 to repair of the mtDNA was studied following CAA exposure. In vitro processing with AAG and APE cleaved the isolated DNA at the site of AAG-specific modified bases. While the contribution of AAG/APE processing is difficult to discern from general alkaline labile sites in mtDNA, the expression of AlkBH7 correlates with increase mtDNA fragment length after 4 hours of recovery. The conclusion that can be drawn from this is that while mtDNA may contain an abundance of alkaline labile sites, expression of AlkBH7 allows more effective recovery following acute treatment with an exogenous agent.

Notably, mtDNA displays similar single-stranded chain length on alkaline gels before and after CAA exposure. The incidence of endogenous alkaline labile sites in mtDNA complicates interpretation of the data. Differences between AlkBH7 expressing lines and the parental HeLa cells are only apparent after recovery. However, the possibility that AlkBH7 activity is modulated by post-translational modification in response to genomic stress may account for this difference. DNA2 has been shown to redistribute to mitochondrial nucleoids following replicative stress (Duxin et al., 2009). AP endonuclease 1 activity in both the nucleus and mitochondria increase with age consistent with the accumulation of oxidation products over time (reviewed in Mitra et al., 2007). It may be interesting to evaluate AlkBH7 activity of mitochondrial extracts prior to and after DNA damage to investigate whether this protein is a target for post translational modification which may modulate the activity. Alternatively, the interaction of the protein with other mitochondrial repair machinery may be modulated as a consequence of DNA damage.

Chapter 6: Transgenic Targeting of a Cytidine Deaminase to Mitochondria to Induce Extensive mtDNA Damage

Introduction

Damage to the mitochondrial genome occurs spontaneously through ROS initiated reactions, deamination of cytosine or incorporation of damaged dNTPs by pol γ. Alternatively, damage can be generated by exogenous agents that can affect all cellular nucleic acids. The consequences of massive mtDNA damage have not been well characterized. It was hypothesized that excessive damage to the mtDNA would accelerate the rate of mutation, decrease the rate of replication and impair cellular viability. To achieve this without inducing cellular stress by introduction of exogenous agents, a strategy was designed that would rely on mitochondrial expression of an enzymatic cytidine deaminase.

The activation- induced cytidine deaminase (AID) is an enzyme that catalyzes the deamination of cytosine into uracil (Bransteitter et al., 2003; Harris et al., 2002). Uracil is recognized and removed from DNA by uracil DNA glycosylases (Caradonna, 1980). Both mitochondrial and nuclear isoforms of UDG exist (Domena, 1985) and respond either to spontaneous deamination of cytosine or misincorporation of UTP or dUTP into the respective genomes. Under normal conditions, AID is a cytoplasmic protein that is imported to the nucleus upon induction of B cell differentiation. Cytosine deamination coupled with BER contributes to class switch recombination and somatic hypermutation thereby adding diversity in the variable regions of the immunoglobulin genes (Schrader et al., 2005). While other cytidine deaminase proteins exist (Harris et al., 2002), AID more

so than other APOBEC enzymes targets ssDNA (Bransteitter et al., 2003) but requires a distinctive sequence context to act (Harris et al., 2002).

In an effort to understand whether high levels of mtDNA damage could affect mtDNA replication, transcription, distribution, cellular function or viability, AID was targeted to mitochondria using the MTS of either pol γB or MnSOD. A catalytically inactive form of AID was generated by mutating an active site cystine which is required for activity in other APOBEC family proteins to alanine. To confirm mitochondrial localization of transgenic mitochondrial AID, DNA constructs encoding these proteins were transfected into HeLa cells and the mitochondria analyzed by immunostaining. Unfortunately, restricted delivery of AID exclusively to mitochondria was not achieved. The experiments in this chapter were only preliminary in nature, but are included here to provide a record of the attempt to use this approach to selectively damage mtDNA.

Materials and Methods

Cloning of Mus musculus AID

A plasmid containing the CDS of AID from Mus musculus was obtained from Dr. Ken Marcu at Stony Brook University. The CDS was amplified by PCR using primers 5'- TTTGGATCCTATGGACAGCCTTCTGA- 3' and 5'-TTTGCGGCCGCAATCCCAACATACGA-3'. The PCR products were cloned into pCR Blunt TOPO (Invitrogen) and sequenced. The CDS was excised from this plasmid and subcloned into mammalian expression vectors containing no MTS, MnSOD-MTS or polγB MTS using BamHI and NotI restriction sites.

Catalytically Inactive AID Constructs

To generate catalytically inactive AID, site directed mutagenesis was used to mutate C87 in AID CDS to A on each of the above constructs. The primers used had the sequence 5'- ACAGTCATAGGCCGGGCTCCA-3' and 5'-TGGAGCCCGGCCTATGACTGT-3'. All constructs were sequenced to confirm the presence of the point mutation.

Transformation and Localization of AID Expression

HeLa cells were plated on 4-well chamber slides and transfected using Fugene 6 HD transfection reagent (Roche) according to manufacturer's protocols. 48 hours after transfection, cells were fixed with methanol, blocked with 0.5% non-fat dairy milk in PBS for 2 hours. Primary antibodies against c-myc, MnSOD or TFAM were applied overnight at 4°C. Each chamber was washed 3 times with PBS and fluorescently labeled secondary antibody was applied for 60 minutes at room temperature with gentle shaking. 3 additional washes with PBS were used to remove unbound secondary antibody, VectaShield was applied and cover slips used to seal the samples. Fluorescence images were captured using standard fluorescein and rhodamine filter sets in the Zeiss LSM510 confocal microscope.

Results

Immunofluorescent detection of MnSOD and Tfam is shown in conjunction with active and catalytically inactive mitochondrially targeted AID in Figure 6.1. In panel A, a construct encoding AID-myc-his shows diffuse cytoplasmic and nuclear staining

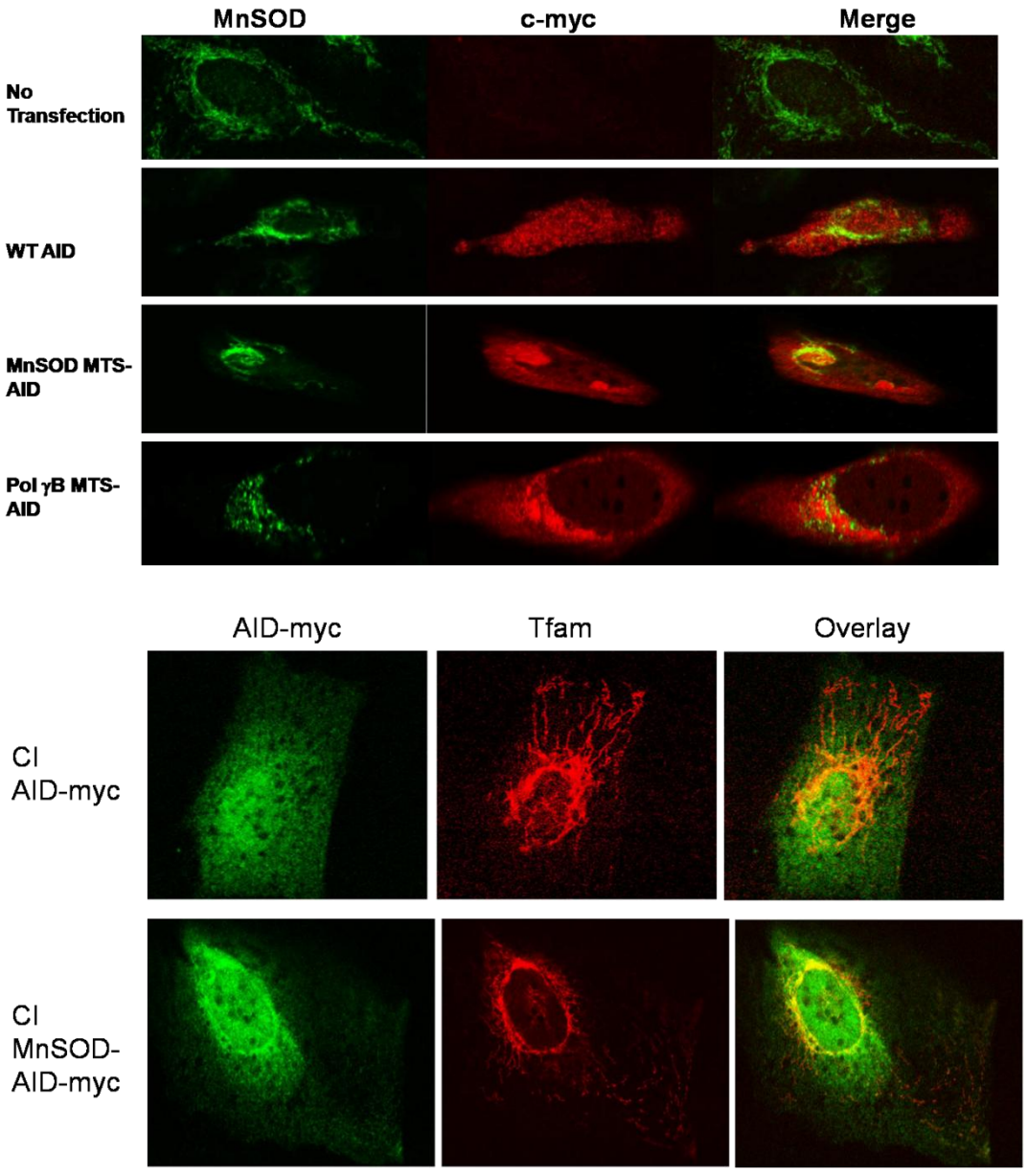


Figure 6.1 Collapse of the mitochondrial reticulum as a consequence of mitochondrial expression of AID. A: Transgenic constructs using the MTS of MnSOD or pol γB were used to target AID mitochondria. AID was detected using a C-terminal myc epitope tag. Mitochondria were detected using MnSOD specific anti-sera. B: Catalytically inactive AID constructs partially colocalize with Tfam. AID was detected using antisera against a c-terminal myc epitope, Tfam was detected using polyclonal antisera.

without appreciable colocalization with MnSOD. Both the MnSOD MTS- and pol γB MTS- AID-myc-his constructs partially colocalize with MnSOD. Complete colocalization is not observed and diffuse cytoplasmic and nuclear staining is maintained despite the presence of the amino-terminal mitochondrial targeting sequences. In panel B, both catalytically inactive AID-myc-his and inactive MnSOD-MTS-AID-myc-his show diffuse cytoplasmic and nuclear localization but the mitochondrially targeted construct has more significant colocalization with Tfam than does the native construct.

Comparing the mitochondrial staining of the MnSOD-AID-myc-his constructs, it is apparent that the mitochondrial reticulum of those cells expressing an active cytidine deaminase has contracted to a perinuclear region while the mitochondrial reticulum of cells expressing an inactive isoform shows a nearly normal distribution. Despite appearances, all cells observed were of normal size and overall morphology and apparent differences in size are due to digital image processing.

Discussion

Complete mitochondrial localization of an AID was not achieved in these experiments. Neither the MnSOD nor the pol γB mitochondrial targeting sequences were sufficient to drive AID completely to the mitochondria. The presence of cryptic subcellular localization signals may have contributed to the incomplete mitochondrial targeting that is observed. Partial mitochondrial localization was achieved. When catalytically active mitochondrial AID was expressed, a compressed perinuclear body with mitochondrial staining was observed. When a catalytically inactive variant of this protein was expressed, the mitochondria maintained more normal distribution. If the mitochondrially targets transgenic protein is active and deaminates cytidine residues in

mtDNA, the collapse of mitochondrial reticulum could be attributed to massive DNA damage which is not observed when inactive enzyme is expressed.

It has been shown that mtDNA replicates in perinuclear areas and that newly replicated mtDNA molecules are distributed throughout the cell (Davis and Clayton, 1996). If perinuclear mitochondria serve as replicative centers this experiment suggests that they might also serve as centers for overall mtDNA maintenance. Theoretically, coupling mitochondrial replication to a region proximate to the nucleus can serve to aid in nuclear: mitochondrial signaling or import of necessary repair factors. Further, mitochondria which maintain a distinct set of dNTP pool enzymes including thymidine kinase (Arnér, 1995), deoxyguanosine kinase (Jüllig, 2000), ribonucleotide reductase (Thelander, 2007), deoxyuridine triphosphatase (Ladner et al., 1996) and mammalian MTH1 (Kang et al., 1995) can provide dNTPs for repair of nuclear DNA during G_0.

Fission and fusion of the mitochondria are essential to maintain mitochondrial function and propagation of the mtDNA. Impairment of mitochondrial fission can lead to mitochondrial dysfunction and loss or propagate mutation of mtDNA (Parone et al., 2008; Malena et al., 2009). Mitochondrial fusion has been shown to occur in response to stresses such as UV irradiation, transcriptional inhibition or inhibition of protein synthesis (Tondera et al., 2009). The collapse of the mitochondrial reticulum observed in this experiment may represent a response to overwhelming damage to the mtDNA brought about by the expression of AID in the mitochondria. When stimulated to undergo apoptosis, mitochondria typically fragment rather than fuse, releasing cytochrome C into the cytoplasm (Suen et al., 2008). The observed phenotype is more indicative of massive mitochondrial fusion than of an apoptotic response.

Mitochondrial dysfunction has been associated with mitochondrial specific autophagic response (mitophagy.) A survey of autophagic literature suggests that general autophagy can occur throughout the cytoplasm of a cell. However, it appears that mitophagy occurs in close location to the nucleus (Kim et al., 2007). Thus it may be the case that in response to overwhelming damage to the mtDNA, the mitochondrial reticulum collapses to a perinuclear complex to attempt to repair the damage. If incapable of repair, mitochondria may be degraded in an autophagic process to prohibit the propagation of dysfunctional mitochondria throughout the cell, thus cellular survival. If this process were to become sustained over time, an overall depletion of cellular energy stores could occur and in an attempt to avoid apoptosis, cellular respiration could shift from oxidative phosphorylation dependent energy production to substrate level phosphorylation. Maintenance of viability would then require increased glucose uptake as observed by Warburg (Warburg, 1924).

Chapter 7: Conclusions and Future Directions

The work presented in this dissertation describes AlkBH7 as a protein component of the human mitochondria which is capable of acting as a direct reversal enzyme to protect the mitochondrial DNA from mutation by endogenous or exogenous agents. While biochemical and cellular activities have been attributed to this protein, in this work, the possibility that AlkBH7 has other roles or biological activities cannot be excluded.

As a DNA repair protein, AlkBH7 demonstrates the ability to complement the activity of AAG in maintaining DNA fidelity. As AAG has a broad range of potential substrates, including the εA substrate tested in this work, the substrate specificity of AlkBH7 may be broader than other AlkB family members. The absence of the long loop of AlkB, AlkBH2 and AlkBH3, which caps the active site and stabilizes interactions with double-stranded DNA, from the primary sequence of AlkBH7 may allow for larger substrates including substituted etheno lesions to be removed by this enzyme. The advantage of employing a direct reversal mechanism for these lesions is that lesions can be processed rapidly without sequestering pol γ from its replicative role and that potentially harmful AP sites and nicks in the phosphate backbone of the mtDNA can be avoided. If LPO lesions are abundant in the mtDNA, and are a normal consequence of respiration, utilizing a direct repair mechanism against these lesions significantly mitigates their consequences.

The increased viability in response to CAA exposure presented in Chapter 5 has some potentially interesting consequences. As briefly discussed, expression of AlkBH7

could mitigate the effect of LPO DNA adducts on the mtDNA, thereby allowing for continued and efficient mitochondrial function over the lifespan of a single cell, tissue, organ or animal. It may be interesting to determine if AlkBH7 polymorphism relates to human disease or expression levels correlate with increased lifespan or cancer incidence. As an endogenous protein that contributes to maintenance of normal mitochondrial function, and as modulation of mitochondrial function by caloric restriction or resveratrol administration mitigate the effect of oxidative damage over the lifespan of rodents resulting in increased lifespan and vitality, it may be of significant interest to study the mechanism of AlkBH7 transcriptional activation and cellular regulation of protein activity.

While the data presented on expression of a mitochondrial cytidine deaminase is limited and does not support any firm conclusions, the observed response raises some interesting questions about the biology of mtDNA replication and repair. Are perinuclear mitochondria distinct from peripheral mitochondria? Are mitochondrial biosynthetic processes organized throughout the cell? Does mtDNA act as a biosensor to determine when a peripheral mitochondrial unit has suffered significant oxidative damage and direct fusion with newly synthesized or rejuvenated mitochondria to mitigate the effect of this damage? Can mitochondrial damage lead to mitophagy and can excessive mitophagy lead to overall changes in the cell? While interesting questions, they are beyond the scope of this dissertation and would require significant time, dedication and energy to test thoroughly.

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