REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

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Bio. Arın DOĞAN

Biochemistry Program
Master of Science Thesis

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ÖZET

Doğan A., İnsan Cockayne sendromu B proteininin NEIL1 enzim aktivitesi üzerine etkileri, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Biyokimya Anabilim Dalı Yüksek Lisans Programı, Ankara, 2008. Cockayne sendromu (CS) erken yaşlanma hastalığıdır ve ultraviyole dalga boyundaki ışığa karşı duyarlılık ile karakterizedir. Gözlenen bu fenotip hastalarda var olan ilerleyici nitelikteki nörodejenerasyonu açıklamaya yeterli degildir. CS fenotipinin oluşmasının başlıca sebebinin CSB proteininden yoksun hücrelerin oksidatif strese karşı gösterdikleri aşırı duyarlılık olduğu düşünülse de, bu fenotipin oluşumuna yol açan moleküler mekanizma halen bilinmemektedir. Bu çalışmanın amacı, CSB proteininin, endonükleaz VIII benzeri DNA glikozilazın (NEIL1) oksidatif hasar onarımı aktivitesinin üzerine etkilerinin arştırılmasıdır. NEIL1 enziminin 2,6-diamino-5-formamidopirimidin (FapyGua); 4,6-diamino-5-formamidopirimidin (FapyAde) ve 5-hidroksi-urasil (5-OH-Ura) içeren substratlar üzerindeki kesim aktivitesinin CSB tarafından uyarıldığı bulunmuştur. Öte yandan, NEIL1 enziminin 7,8-dihidroksi-8-oksoguanin (8-OH-Gua) içeren substrata yönelik kesim aktivitesinin CSB tarafından uyarılmadığı saptanmıştır. CSB proteininin, NEIL1 enziminin AP-liyaz (zincir kırma) aktivitesini doğrudan uyardığı saptanmıştır. CSB proteinin varlığında, NEIL1 enziminin bazı kesme işlevinin zincir kırma işlevinden kenetsizlenmediği bulunmuştur. Bu bulgular CSB proteinin, NEIL1 enzimiyle ortak çalışarak baz kesip-çıkarma onarımı mekanizmasında görev aldığını göstermektedir. Buna ek olarak onarılamayan ya da yanlış onarılan FapyAde ve FapyGua DNA hasarlarının Cockayne sendromunun patolojisine katkısı olabileceği düşünülmektedir.

Anahtar kelimeler: Cockayne Sendromu, CSB proteini, NEIL1 DNA glikozilaz, baz kesip-çıkarma onarımı, oksidatif DNA hasarı.

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ABSTRACT

Doğan A., The Effects of Human Cockayne Syndrome B Protein on the NEIL1 Enzyme Activities, Hacettepe University Institute of Health Sciences Department of Biochemistry, MSc thesis, Ankara, 2008. Cockayne syndrome (CS) is a premature aging syndrome characterized by UV sensitivity. However, this phenotype does not explain the progressive neurodegeneration in CS patients. CS is caused by mutations in *CSA* and *CSB* genes. The role of CSB in molecular mechanisms underlying CS phenotypes is unclear. That could be due to the hypersensitivity of CSB deficient cells to oxidative stress. The aim of this study is to investigate the effects of CSB on endonuclease VIII-like DNA glycosylase (NEIL1) enzyme activity to repair oxidative DNA lesions. It was found that CSB stimulates NEIL1 incision activity on 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 4,6-diamino-5-formamidopyrimidine (FapyAde) and 5-hydroxy uracil (5-OH-Ura)-contaning substrates. However, CSB did not stimulate the incision activity of NEIL1 on 7,8-dihydro-8-oxoguanine (8-OH-Gua)-containing substrate. It was also found that CSB direclty stimulates AP-lyase (strand cleavage) activity of NEIL1, and the base release by NEIL1 is not uncoupled from strand cleavage, either in presence or absence of CSB. These findings suggest that CSB is involved in base excision repair of oxidative DNA lesions in cooperation with NEIL1 DNA glycosylase. In addition, unrepaired or misrepaired oxidative DNA lesions such as FapyAde and FapyGua may contribute to the pathology of CS.

Keywords: Cockayne syndrome, CSB protein, NEIL1 DNA glycosylase, base excision repair, oxidative DNA lesions

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SYMBOLS AND ABBREVIATIONS

5-OH-Ura	5- hydroxyl uracil
8-OH-Gua	7,8-dihydro-8-oxoguanine
A	Adenine
APE1	Apurinic/apyrimidinic endonuclease 1
Arg	Arginine
ATP	Adenosine triphosphate
BER	Base excision repair
CNS	Central nervous system
DNA	Deoxyribonucleic acid
BSA	Bovine serum albumin
C	Cytosine
CPD	Cyclobutane pyrimidine dimer
CS	Cockayne syndrome
CSA	Cockayne syndrome group A protein
CSB	Cockayne syndrome group B protein
dRP	5'-2' deoxyribose phosphate
dsDNA	Double strand DNA
EDTA	Ethylenediaminetetraacetic acid
ERCC6	Excision repair cross-complementing rodent repair deficiency complementation group 6
ERCC8	Excision repair cross-complementing rodent repair deficiency complementation group 8
Fapy	Formamidopyrimidine
FapyAde	4,6-diamino-5-formamidopyrimidine
FapyGua	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FPG	Formamidopyrimidine-DNA glycosylase
G	Guanine
G-25	Sephadex G25 resin

H2TH	Helix-two turn-helix motif
HA-tag	Hemaglutinin antigen
His	Histidine
hmdU	5-Hydroxymethyluridine
IR	Ionizing radiation
Lig III	Ligase 3
LigI	Ligase I
MMS	Methyl methanesulfonate
MPG	N-methylpurine-DNA glycosylase
MYH	A/G-specific adenine DNA glycosylase
NA-AAF	N-acetoxy-2-acetylaminofluorene
Nei	Endonuclease VIII
NEIL1	Nei-like endonuclease 1, Endonuclease VIII-like 1
NLS	Nuclear locator sequence
Nonidet P40	Non-ionic detergent P40
nt	Nucleotide
NTB	Nucleotide binding domain
NTH1	Endonuclease III-like protein 1
OGG1	8-oxoguanine DNA-glycosylase 1
PARP-1	Poly (ADP-ribose) polymerase family member 1
PCNA	Proliferating cell nuclear antigen, FEN1 flap structure-specific endonuclease 1
PNK T4	Polynucleotide Kinase T4
pol β	DNA polymerase β
PUA	4-hydroxypentenal phosphate
Rbx1	Ring-box 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SMUG1	Single-strand-selective monofunctional uracil-DNA glycosylase 1

SSB	Single strand break
ssDNA	Single strand DNA,
SWI/ SNF2	Switching (yeast mating type) / Sucrose NonFermentable 2
T	Thymine
TCR	Trancsription coupled repair
TDG	Thymine-DNA glycosylase
TFIIH	Transcription factor II H
Tg	Thymine glycol
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
UNG	Uracil-DNA glycosylase
UV	Ultraviolet
UV-61	Hamster homologue of human Cockayne syndrome (CS) group B cells
WT	Wild type
XP/CS	Xeroderma Pigmentosum/ Cockayne Syndrome Phenotype
XPB	Xeroderma Pigmentosum group B
XPD	Xeroderma pigmentosum group D
XPG	Excision repair cross-complementing rodent repair deficiency, complementation group 5
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells1

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1. INTRODUCTION

Reactive oxygen species (ROS) are formed in living cells by normal cellular metabolism and by exogenous sources such as ionizing radiation (IR), hydrogen peroxide (H_2O_2) and genotoxic agents. These species cause oxidative damage to DNA such as base modifications. Accumulation of oxidative DNA modifications [7,8-dihydro-8-oxoguanine (8-OH-Gua), 7,8-dihydro-8-oxoadenine (8-OH-Ade) and formamidopyrimidines (2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine)] have been associated with aging, neurodegeneration and carcinogenesis (de Souza-Pinto et al., 2002; Loft et al., 1996; Mattson et al., 2002). Oxidatively induced DNA damage is mainly repaired by base excision repair (BER) pathway, which involves lesion-specific DNA glycosylases in the first step of the repair process. The substrate specificities of DNA glycosylases are broad and, in some cases, overlapping. 8-oxoguanine DNA glycosylase (OGG1) is specific for the repair of 8-OH-Gua and FapyGua oxidative DNA lesions. FapyGua (2,6-diamino-4-hydroxy-5-formamidopyrimidine), FapyAde (4,6-diamino-5-formamidopyrimidine), 5-OH-Ura (5-hydroxyl uracil) and 8-OH-Gua are substrates for endonuclease VIII-like (NEIL1) DNA glycosylase (Das et al., 2005; Dou et al., 2003; Hazra et al., 2002).

There are several human genetic diseases which cause a defect in one of the DNA repair systems. Defects in nucleotide excision repair (NER) pathway cause mainly three human premature aging disorders; Xeroderma pigmentosum (XP), Cockayne syndrome (CS) and Trichothiodystrophy (TTD) (Licht et al., 2003). CS is an autosomal recessive disorder characterized by many neurological and developmental abnormalities. Eighty percent of the CS cases are caused by a defect in the *CSB* gene while the remaining cases are caused by mutations in the *CSA* gene (Troelstra et al., 1992). The CSB protein (CSB) belongs to SWI/SNF2 family of proteins that have conserved ATPase/putative helicase motif like other members of this family (Troelstra et al., 1992). CSB has two distinct biochemical activities which are ATPase and single strand DNA annealing activities (Citterio et al., 2002; Muftuoglu et al., 2006; Selby and Sancar, 1997a).

A hallmark of CSB deficient cells is the sensitivity to ultraviolet radiation (UV) induced DNA damage. Normally, UV-lesions (cyclobutane pyrimidine dimers (CPD) and 6-pyrimidine-4-pyrimidone products) are preferentially removed from the transcribed strand of actively transcribed regions of the genome by the so-called transcription coupled repair (TCR), which is a sub-pathway of NER (Bohr et al., 1985). This pathway does not function in CSB deficient cells, indicating that CSB plays an important role in TCR (Licht et al., 2003; Venema et al., 1990). In addition, CSB has a role in chromatin remodeling and regulation of transcription (Balajee et al., 1997; Citterio et al., 2002; Dianov et al., 1997). However, these biochemical and cellular properties do not directly explain the progressive neurodegeneration observed in the CS patients. It has been proposed that neurological symptoms in CS may be due to defective repair and/or processing of oxidative DNA damage in CSB deficient cells (Stevnsner et al., 2008).

Cellular studies demonstrated that mouse embryonic fibroblasts from CSB knockout mice, primary and SV-40 transformed CSB deficient fibroblasts are hypersensitive to oxidative DNA damaging agents such as IR, NA-AAF and paraquat (de Waard et al., 2003; 2004; Leadon et al., 1993; Tuo et al., 2001). Moreover, oxidative DNA lesions (8-OH-Gua and 8-OH-Ade) accumulates in CSB deficient cells after oxidative stress, indicating that CSB may play a role in the repair of these lesions (Tuo et al., 2001; 2003). More importantly, hepatocytes, splenocytes and kidney cells from OGG1/CSB double knockout ($OGG1^{-/-}/CSB^{-/-}$) mice accumulate several-fold higher levels of 8-OH-Gua than $CSB^{-/-}$ mice and have elevated levels of G to T transversion mutations (de Waard et al., 2003; 2004).

Since CSB has a role in TCR, it has been hypothesized that oxidative DNA damage may be repaired by TCR. It is argued that the TCR deficiency of oxidative damage could be responsible for the progressive neurological disorders seen in CS patients (de Waard et al., 2003; Laposa et al., 2007; Osterod et al., 2002; Pastoriza-Gallego et al., 2007). This area of study has been filled with controversy. For the initiation of TCR, RNA polymerase II has to be arrested when it encounters a lesion located in a transcribed strand of active gene. However, oxidative DNA lesions e.g. 8-OH-Gua do not block RNA polymerase II during transcription (Kathe et al., 2004; Larsen et al., 2004; Tornaletti et al., 2004; Viswanathan and Doetsch, 1998). RNA

polymerase II can bypass an 8-OH-Gua lesion, which is a nonbulky lesion repaired generally by BER process. Thus, CSB might have additional functions outside of TCR, possibly in BER pathway to repair oxidative DNA damage.

The reduced repair of 8-OH-Gua in CSB deficient cells is associated with a down regulation of OGG1 protein (Dianov et al., 1999; Tuo et al., 2002). CSB is found in a complex with OGG1, although no direct interaction between the two proteins have been identified (Stevnsner et al., 2002; Tuo et al., 2002). In addition to accumulation of 8-OH-Gua and 8-OH-Ade in CSB deficient cells, it has recently been demonstrated that FapyGua and FapyAde oxidative lesions significantly accumulate in brain, liver and kidney DNA of CSB knockout mice (Muftuoglu et al., unpublished data). The incision of these lesions is catalyzed by NEIL1 DNA glycosylase (Hazra et al., 2002). NEIL1 knockout mice develop a combination of clinical manifestations resembling human metabolic syndrome as it is observed in CS patients (Vartanian et al., 2006). Thus, elevated levels of FapyGua and FapyAde may play a role in the pathophysiology of CS. Since, FapyGua and FapyAde accumulate in CSB knockout mice tissues (Muftuoglu et al., unpublished data), this study hypothesizes that CSB may play a role in the repair of formamidopyrimidines together with NEIL1 DNA glycosylase in BER pathway. CSB may affect the catalytic activities of NEIL1 in this repair process.

2. BACKGROUND

2.1. Cockayne Syndrome

2.1.1. Clinical Characteristics of Cockayne Syndrome

Cockayne syndrome is a rare autosomal recessive genetic disease and classified as a segmental premature aging syndrome. It was first reported in 1936 by Dr. Edward A. Cockayne. Cockayne syndrome has a diverse clinical phenotype including severe impairment of physical development, cachectic dwarfism, progressive neurological degeneration, white matter hypomyelination and central nervous system (CNS) calcification, sensorineural hearing loss, sunken eyes, lack of subcutaneous fat and cataracts. Cockayne syndrome patients are hypersensitive to UV light but they do not show cancer predisposition. The life expectancy of patients with CS is approximately 12 years (Licht et al., 2003).

2.1.2. Genetics of Cockayne Syndrome

Two genes are responsible from the CS phenotype, CS complementation group B (*CSB*) gene (also called excision repair cross complementing; *ERCC6*) and *CSA* (also called *CNK1* or *ERCC8*) gene. In 80% of the cases, CS is caused by a defect in *CSB* gene. The mutations of genes for xeroderma pigmentosum (*XPB*, *XPD*, and *XPG*) result in combined XP/CS phenotype (Licht et al., 2003).

The *CSA* gene is located on chromosome 5q12—q13, it was first cloned in 1995 (Henning et al., 1995). *CSA* gene encodes CSA protein (CSA), which has a molecular weight of 44 kDa and consists of 396 amino acids. The CSA protein belongs to the "WD repeat" family of structural and regulatory proteins that lack enzymatic activity. This protein is part of a multisubunit ubiquitin ligase complex, containing Cullin 4A, Roc 1 (Rbx1), and DNA damage binding protein 1 (DDB1) (Entrez gene).

The *CSB* gene (also known as *ERCC6* gene) is located on chromosome 10q11. *CSB* gene encodes a protein of 1493 amino acids with a molecular weight of 168 kDa. The CSB protein belongs to SWI/SNF2 family of proteins. Cockayne syndrome B protein consists of an acidic amino acid strech, a glycine-rich region,

two putative nuclear localization signals and seven conserved ATPase motifs I, Ia, II, III, IV, V, and VI (Entrez gene; Licht et al., 2003; Troelstra et al., 1992) (Figure 2.1).

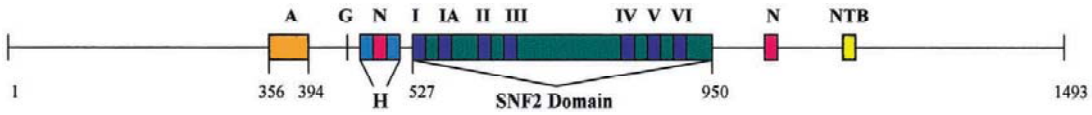

Figure 2.1. Predicted motifs of CSB. A: Acidic domain. G: Glycine-rich region. H: Hydrophilic region. N: Bipartite nuclear localization signal. I-VI: ATPase motifs located in the SNF2 domain conserved among the members of the SWI/SNF2-like family. NTB: Putative nucleotide binding motif (Licht et al., 2003).

The severity of the disease does not seem to correlate with the site or nature of the CSB mutation. This suggests that the genetic background and/or environmental factors may be involved in determining the specific pathological phenotype of CS. The downstream targets of the CSB protein-dependent regulation may also be involved (Licht et al., 2003; Stevnsner et al., 2008).

2.2. Biochemical Characteristics of CSB

Although no helicase activity has been ascribed to CSB, the protein possesses a DNA-dependent ATPase activity, and a single stranded DNA annealing activity (Citterio et al., 2002; Muftuoglu et al., 2006; Selby and Sancar, 1997a). The ATPase domain is required for CSB function in chromatin remodeling and modulation of negative DNA supercoiling (Citterio et al., 2002). Chromatin remodeling activity of CSB requires ATP hydrolysis, but the ssDNA annealing activity of CSB does not. Interestingly, ATP binding inhibits ssDNA annealing by CSB (Muftuoglu et al., 2006). The biological relevance of this activity is not yet clear, but is presumably associated with DNA repair, possibly recombinational repair. An earlier described function of CSB is in the TCR of UV induced DNA lesions (cyclobutane pyrimidine dimers, and 6-pyrimidine-4-pyrimidone) products. Recently, evidence suggests a potential role for the CSB protein in BER of oxidative DNA lesions (Licht et al., 2003). Stably transformed human cell lines with site-directed mutations of the *CSB*

gene in specific helicase domains were generated by replacing the highly conserved amino acids to help understand the role of different conserved helicase motifs of CSB in oxidative DNA damage response (Muftuoglu et al., 2002; Tuo et al., 2001; 2003). However, its more precise function in BER has not yet been investigated.

The enzymatically active CSB protein functions as a homodimer, and the dimerization occurs through the central ATPase domain of the protein (Christiansen et al., 2005). Using atomic force microscopy for analysis of CSB-DNA complexes, Beerens and co-workers (Beerens et al., 2005) demonstrated that DNA wraps around dimers of CSB. DNA wrapping and unwrapping may allow CSB to actively alter the DNA double helix conformation, which could influence nucleosomes and other protein-DNA interaction (Beerens et al., 2005).

2.2.1. CSB in TCR and Transcription

An important phenotypic characteristic of CSB cells is the deficient recovery of RNA synthesis after exposure to UV. Transcription in CS cells is inhibited following UV exposure, while normal cells show a recovery up to 90% of the pretreatment levels, CS cells fail to recover (Mayne et al., 1984). This is an indicator of a defect in TCR (Bohr et al., 1985; Troelstra et al., 1992). CSB binds RNA polymerase II when arrested at a template lesion *in vitro* and promotes recruitment of TFIIH.

CSB has a role in the regulation of transcription. DNA lesions caused by N-acetoxy-2-acetylaminofluorine blocks the elongation of RNA strand by RNA polymerase II. This was caused by their inability to recover RNA synthesis after NA-AAF. This suggests that the sensitivity of CS cells to the genotoxic agent NA-AAF is caused by a defect in transcription, probably in re-initiation of transcription (van Oosterwijk., 1996).

In 1997, Selby and Sancar demonstrated *in vitro* that CSB mildly stimulated transcription elongation on naked DNA. CSB is involved in transcription by RNA polymerase I, II and possibly III. In 1997, Balajee et al. have shown a 50% reduction in transcription in CSB fibroblasts and lymphoblasts. This defect was associated with flawed elongation and transcription. In 2002, Bradsher et al. showed a reduced

rRNA synthesis in CSB cells and a 10-fold stimulation of rRNA synthesis in the presence of a recombinant form of CSB protein *in vitro*.

2.2.2. CSB in Chromatin Remodeling

CSB is the first DNA repair protein that is found to function directly in modulating nucleosome structure. Formation of chromatin structures inhibits various processes such as transcription, recombination and DNA repair. CSB can alter DNA conformation and induce changes in chromatin structure in an ATP dependent manner. The partial decondensation of chromatin structure by CSB may provide access to DNA damage processing factors (Citterio et al., 2002).

The capability of CSB to modulate DNA double helix conformation may directly facilitate TCR. CSB is suggested to be involved in opening of chromatin to facilitate displacement of stalled RNA polymerase II-DNA complex and enable the NER enzymes to access the damage, rapid refolding of nucleosomes (Moggs and Almouzni, 1999). The displacement of stalled RNA polymerase II-DNA by CSB may facilitate the TCR process (Citterio et al., 2002).

2.2.3. CSB in Apoptosis

CSB cells are predisposed to undergo apoptosis in response to UV treatment (Ljungman and Zhang, 1996). This property of CSB deficient cells explains the absence of skin cancer development in CS patients (Nickoloff and Hoekstra, 1998). Induction of apoptosis by using UV is associated with accumulation of active p53 and inhibition of total RNA synthesis (Ljungman and Zhang, 1996). A mutated *CSB* gene is directly responsible for the tendency to UV-induced apoptosis of CSB deficient cells (Balajee and Bohr, 2000). CSB has been suggested to function as an anti-apoptotic factor by preventing the blockage of RNA polymerase II transcription by UV-induced DNA damage in normal cells (Balajee and Bohr, 2000; Ljungman and Zhang, 1996). CSB cells show a higher and longer lasting induction of p53 at low UV doses than in normal human fibroblasts. UV-induced apoptosis can be independent of p53 (Spivak et al., 2003) as suggested by the increased tendency towards UV-induced apoptosis in UV-61 which has a mutant form of p53. Progression into the S phase is necessary to induce apoptosis (Proietti de Santis et

al., 2002). Because of their defected TCR, at low doses of UV, CSB deficient cells accumulate more damage than normal cells and show a greater tendency to undergo apoptosis following the S phase. On the other hand, at higher doses, the damage could be so high that CSB deficient cell's entry into S phase is inhibited and the apoptosis amount is reduced compared to the normal functioning cells which repair the accumulated damage and then proceed to the S phase followed by apoptosis (McKay et al., 2002).

2.3. CSB and Oxidative DNA Damage

Cellular studies have demonstrated accumulation of oxidative DNA damage in CSB deficient cells after oxidative stress. This implies that there might be an important potential role for CSB in BER (Tuo et al., 2002; 2003). In order to understand the molecular mechanisms that might explain the role of CSB in the repair of oxidative lesions, several different biochemical approaches were used. Dianov et al., (1999) made the first demonstration that CSB mutant cells are defective in the incision of 8-OH-Gua. The reduced repair of 8-OH-Gua is associated with a downregulation of human OGG1 gene expression and protein level in CSB mutant cells (Dianov et al., 1999; Tuo et al., 2002). This deficiency is complemented by transfection of CSB mutant cells with the normal *CSB* gene (Dianov et al., 1999). On the other hand, it was shown that CSB status does not affect the incision activities of two other DNA glycosylases, thymine glycol DNA glycosylase (hNTH) and uracil DNA glycosylase (hUDG). CSB mutant cells are also defective in the repair of 8-OH-Ade, another abundant lesion in oxidatively damaged DNA. This indicates that CSB might be one of the factors important for the repair of 8-OH-Ade (Muftuoglu and Bohr, 2008). The glycosylase involved specifically in the repair of this lesion has not yet been identified (Jensen et al., 2003). Furthermore, cell extracts from stably transformed human cell lines with site directed CSB mutations in various ATPase domains have shown that ATPase domains V and VI of CSB are important for the role of the protein in the processing of 8-OH-Gua lesions (Muftuoglu et al., 2002; Tuo et al., 2001) whereas only domain VI appears to be involved in the repair of 8-OH-Ade (Tuo et al., 2002).

In humans, OGG1 exists in both nuclear and mitochondrial (mtOGG1) isoforms. The mtOGG1 protein level is low in CSB-deficient cells. Stevnsner et al., in 2002, showed a reduced 8-OH-Gua incision activity in both the mitochondrial extracts of CSB deficient cells and that of CSB knockout mouse liver cells, indicating a potential role for CSB also in the mitochondrial repair of oxidative base damage (Stevnsner et al., 2002; 2008). This activity of the CSB protein in mitochondria is specific for the repair of 8-OH-Gua, since CSB deficient cells have normal levels of uracil, thymine glycol and hypoxanthine incision activities (Stevnsner et al., 2002). Mitochondrial BER plays a crucial role in protecting the integrity of mitochondrial DNA (mtDNA). Since mitochondria are the primary source of endogenous ROS, the accumulation of oxidative DNA damage and mutations in mtDNA may lead to mitochondrial dysfunction and to cell death (Ames et al., 1995).

Evidence shows that CSB may have a role in BER in nuclei and mitochondria. Recently, CSB has been shown to interact physically and/or functionally with more proteins involved in the BER pathway including poly(ADP-ribose) polymerase 1 (PARP-1) and APE1 (Flohr et al., 2003; Thorslund et al., 2005; Wong et al., 2007). PARP-1 is a nuclear enzyme that protects the integrity of the genome by responding to oxidative DNA damage and facilitating DNA repair. CSB interacts with PARP-1 and is found at PARP-1 sites after oxidative damage (Thorslund et al., 2005). It has been suggested that PARP-1 stimulation of BER depends on CSB. ATPase activity of CSB is not required for this because poly(ADP-ribosyl)ation of CSB inhibits its DNA-dependent ATPase activity (Muftuoglu and Bohr, 2008).

It has also been shown that CSB physically interacts with APE1 protein and stimulates its activity in a concentration dependent manner. CSB mutant cell lines were hypersensitive to DNA damage induced by an alkylating agent methyl methanesulfonate (MMS) and a base analog, 5-hydroxymethyl-2'-deoxyuridine (hmdU) which introduce base excision repair intermediates (Wong et al., 2007).

2.4. Oxidative DNA Damage

Reactive oxygen species are ubiquitous oxidizing agents that are generated in all organisms either by endogenous or exogenous sources. Endogenous ROS, in the forms of superoxide, hydrogen peroxide, and hydroxyl radicals, are produced as by-products of normal mitochondrial respiration, and are also produced as a result of the metabolism of toxic agents or induced during inflammatory responses. Exogenous sources for ROS include IR, UV, and chemical agents (Dizdaroglu, 2005). ROS damages lipids, proteins and nucleic acids in the cell. Among them, DNA damage is the most important since it can induce mutagenic outcome. Accumulation of oxidative DNA damage is implicated in the etiology of many diseases, including neurodegenerative disorders, cancer and aging (de Souza-Pinto and Bohr, 2002; Loft and Poulsen, 1996; Mattson and Liu, 2002; Wilson and Bohr, 2007). To maintain the integrity of genetic material, cells possess multiple DNA repair pathways such as BER pathway. BER functions in the repair of mainly oxidative DNA damage (Nelson and Cox, 2005; Wilson and Bohr, 2007).

ROS generate numerous DNA lesions such as modified bases and sugar moieties, strand breaks, and DNA-protein cross-links. Currently, more than 20 different oxidatively modified bases have been identified (Figure 2.2) (Evans et al., 2004). Among them, 7,8-dihydro-8-oxoguanine lesion is the most common DNA lesion. In the literature, this base lesion is variously referred to as 8-hydroxyguanine and 8-oxoguanine; this reflects its keto-enol tautomerism with the latter predominating under physiological conditions (Evans et al., 2004). 8-OH-Gua is a mutagenic DNA lesion since it can mispair with adenine during DNA replication, thus producing G:C to T:A transversions (Grollman and Moriya, 1993). *In vivo* measurements indicate that insertion of an A opposite 8-OH-Gua occurs in human cells at a frequency of 17% of replication events (Avkin and Livneh, 2002). 8-OH-Gua forms at a rate of approximately 1000 lesions per cell per day (Kunkel, 1999). 8-OH-Gua is used as a biomarker of oxidative DNA damage in organisms (Grollman and Moriya, 1993).

Although uracil is normally confined to RNA, one of the ways it arises in DNA is by the deamination of cytosine. One of the factors enhance the deamination

of cytosine is oxidative stress. The deamination of cytosine oxidation products can yield uracil derived lesions in DNA. For example, deamination and dehydration of cytosine glycol (Figure 2.2) gives uracil glycol, 5-hydroxycytosine, and 5-hydroxyuracil (5-OH-Ura) (Evans et al., 2004). All these compounds arise due to endogenous DNA damage and exposure of gamma irradiation, indicating that they simultaneously exist in damaged DNA (Cooke et al., 2003). 5-OH-Ura is highly mutagenic since it causes C to T transitions (Kreutzer and Essigmann, 1998).

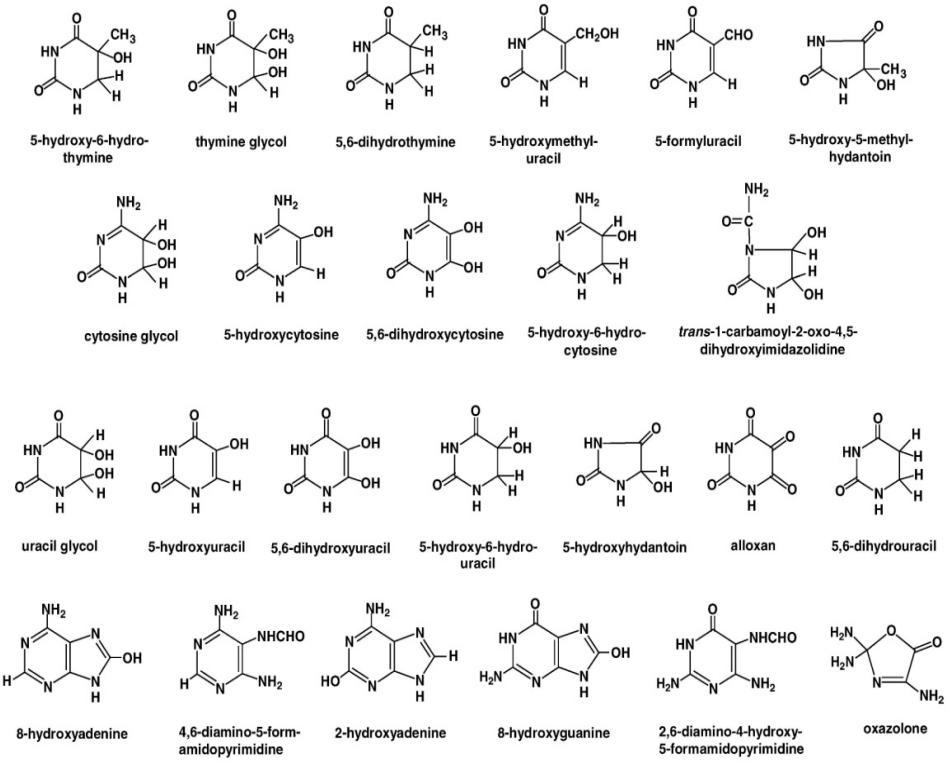

Figure 2.2. DNA base products detected in free radical damaged DNA (Evans et al., 2004).

Formamidopyrimidines (Figure 2.2), i.e., 2,6-diamino-4-hydroxy-5-formamido-pyrimidine and 4,6-diamino-5-formamidopyrimidine are major products of hydroxyl radical attack on guanine and adenine respectively (Evans et al., 2004). These lesions are formed abundantly in DNA after exposure to IR or to other free radical-generating systems. Formation of formamidopyrimidines in DNA upon UV exposure has also been reported (Augeri et al., 1997). *In vitro* studies indicate that these lesions are miscoding and can block the progression of DNA polymerases (Hazra et al., 2000). Several DNA glycosylases that recognize and repair the modified DNA lesions have been identified. For example, 8-oxoguanine DNA glycosylase (OGG1) is the major DNA glycosylase for 8-OH-Gua in humans (Hu et al., 2005).

2.5. Base Excision Repair in Mammalian Cells

BER is the primary pathway for repairing some of the most common DNA lesions including oxidized, alkylated, deaminated or hydrolyzed bases as well as single strand breaks (SSBs) (Nilsen and Krokan, 2001). BER is divided into two subpathways, (a) short-patch or (b) long-patch BER (Figure 2.3) (Hegde et al., 2008). In short-patch BER, a single nucleotide is incorporated whereas in long-patch BER several nucleotides are incorporated during the repair process. The first step in both short- and long-patch BER is the removal of the damaged base (e.g. 8-OH-Gua) by lesion-specific DNA glycosylases. These enzymes recognize a damaged base and hydrolyze the N-glycosidic bond between the base and the deoxyribose sugar to release the base. The initial product of a DNA glycosylase is an abasic or apurinic/apyrimidinic (AP) site in DNA (Hegde et al., 2008; Nilsen and Krokan, 2001).

Second step requires the AP lyase activity to cleave the phosphodiester backbone near the AP site. After an intact AP site is generated by monofunctional DNA glycosylases, APE1 incises the phosphodiester bond 5' to an AP site and creates a single strand break with 3' hydroxyl (OH) end and an abnormal 5'-2' deoxyribose phosphate (dRP). On the other hand, some DNA glycosylases such as OGG1 and the endonuclease VIII-like protein, are bifunctional enzymes with intrinsic AP-lyase activity. The enzyme lyase activity catalyzes either β-elimination

of the 3' phosphodiester bond or β,δ-elimination of the 3' and 5' phosphodiester bonds, depending on the enzyme (Figure 2.3) (Hegde et al., 2008). Subsequently, APE1 removes the 3' terminal 4-hydroxypentenal phosphate (PUA; formed by β-elimination) or a polynucleotide kinase (PNK) removes the 3' terminal phosphate (formed by β,δ-elimination) that block repair synthesis in human cells (Hegde et al., 2008; Kulkarni and Wilson, 2008).

Gap filling repair at the strand break, can then proceed through short or long-patch BER. In short-patch BER, DNA polymerase β (pol β) adds a single nucleotide to the newly generated 3'-OH and removes the 5'-dRP group. Then DNA Ligase III, which interacts with pol β through the X-ray cross-complementing 1 (XRCC1) protein, seals the nick to restore the original DNA sequence which finalizes the process (Hegde et al., 2008; Kulkarni and Wilson, 2008; Sung and Demple, 2006).

When the AP sites are further oxidized or reduced, pol β cannot remove the APE1 generated 5' terminus via its dRP lyase activity, and long-patch BER takes place to promote strand displacement. Long-patch BER involves the proliferating cellular nuclear antigen (PCNA)-dependent polymerases, i.e. pol ε or pol δ, and pol β also be involved. These polymerases synthesize 2–10 nucleotides by displacing the downstream DNA strand. Flap endonuclease 1 (FEN1) cleaves the displaced 5'-flap and DNA Ligase I seals the nick to complete repair (Hegde et al., 2008; Kulkarni and Wilson, 2008).

2.6. DNA Glycosylases

To date, more than eight different DNA glycosylases have been identified in mammals (Table 2.1) (Hegde et al., 2008; Huffman et al., 2005). Mechanistically, they are divided into two groups: monofunctional DNA glycosylases and bifunctional DNA glycosylases/AP lyases. A distinction between these two types of enzymes is that monofunctional DNA glycosylases cleave the glycosidic bond using a water molecule as a nucleophile to generate intact AP site whereas bifunctional glycosylases/AP lyases use ε-NH_2 of a lysine or the N-terminal proline as a nucleophile to form a Schiff base intermediate with β-elimination or β,δ-elimination reactions (Figure 2.4). Usually the excision of damaged base and lyase reaction steps act in a concerted sequence. However, in some cases, such as for OGG1, the lyase

reaction is very weak. Thus intact AP sites are the major product after OGG1-catalyzed cleavage of 8-OH-Gua from DNA (Hegde et al., 2008; Huffman et al., 2005).

DNA glycosylases specific for the repair of oxidized base lesions are bifunctional. On the other hand, monofunctional glycosylases are specific for repair of alkylated bases. Although these enzymes show distinct substrate specificities, there is a considerable degree of overlap, most notably among DNA glycosylases that repair oxidative DNA damage. For example, the OGG1 protein is the major DNA glycosylase involved in the excision of 8-OH-Gua, and endonuclease III (NTH1) is the major DNA glycosylase involved in the excision of oxidized pyrimidines, such as 5-hydroxycytosine, 5-OH-Ura, and thymine glycol (Tg). 5-OH-Ura, Tg and 8-OH-Gua are also substrates for NEIL1 enzyme. In addition, FapyGua lesions are repaired by NEIL1, NTH1 and OGG1. Because of this overlap, the inhibition or elimination of one repair activity does not necessarily render a particular base lesion unrepairable. However, some repair activities may be regarded as back-up mechanisms and are often less efficient than the primary repair pathway and may only operate to a minimal extent when primary repair processes are fully functional (Fromme et al., 2004; Hegde et al., 2008; Huffman et al., 2005).

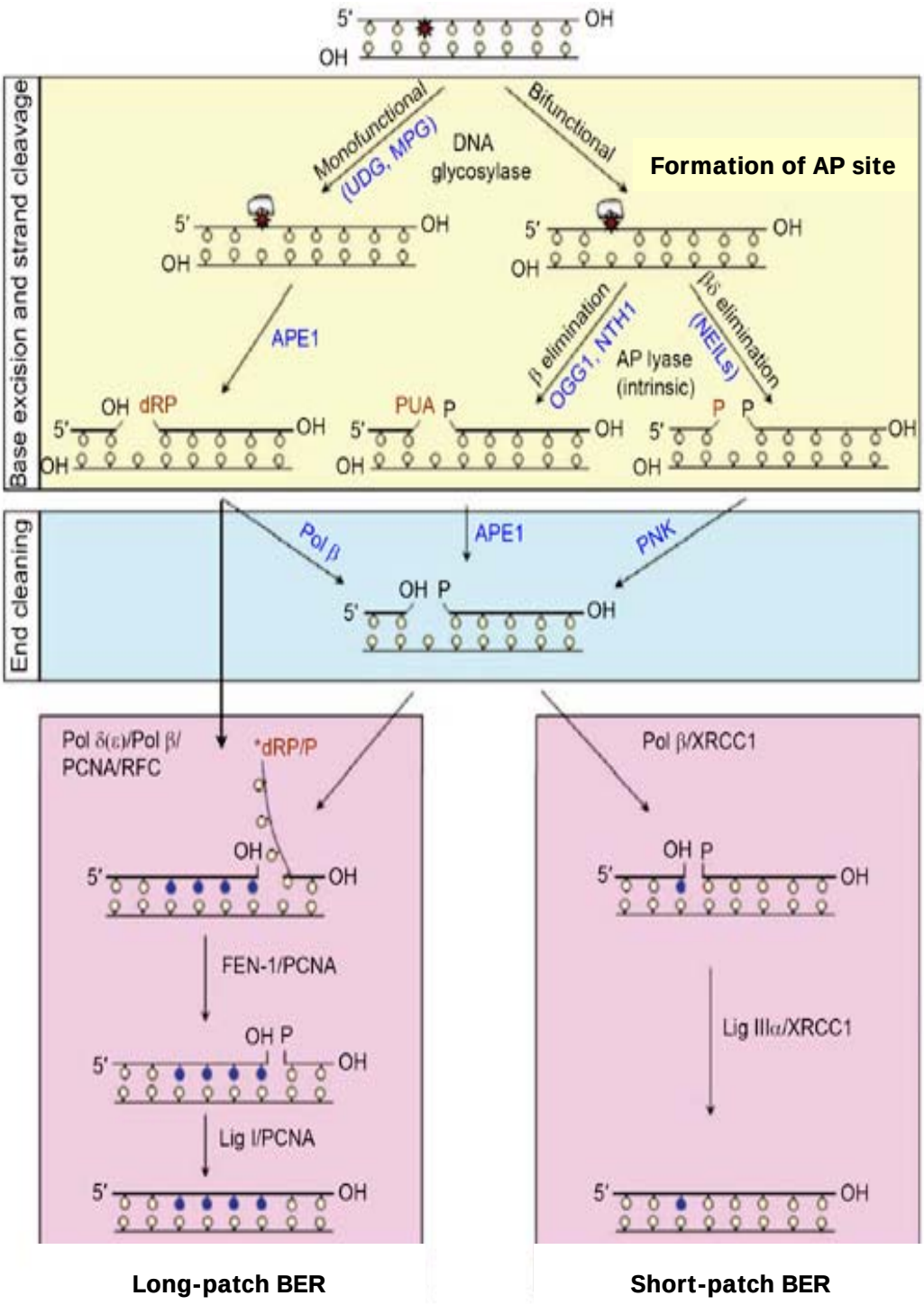

Figure 2.3. A schematic illustration of BER subpathways (Hegde et al., 2008).

Table 2.1. Mammalian DNA glycosylases.

Enzyme	Protein	Type	Preferred substrate
Uracil DNA glycosylase (UDGs)	UNG	Monofunctional	Uracil
	SMUG1		Uracil
	TDG		U and T in U(T)·G
Alkylbase DNA glycosylase	MPG	Monofunctional	3-methyladenine, Hypoxanthine
Adenine specific mismatch DNA glycosylase	MYH	Monofunctional	A in 8-OH-Gua.Ade
DNA glycosylases for oxidized bases	NTH1	Bifunctional (β)	Tg, 5-formylU
	OGG1	Bifunctional (β)	8-OH-Gua, FapyGua
	NEIL1	Bifunctional (βδ)	FapyAde, FapyGua, Tg, 5-OH-Ura
	NEIL2	Bifunctional (βδ)	5-OH-Ura

Bifunctional glycosylases, which forms a transient Schiff base intermediate, can be trapped by the addition of a reducing agent such as $NaBH_4$ or $NaCNBH_3$. Once covalently bound, trapped molecules no longer participate in the overall reaction resulting in stable glycosylase–DNA complexes (Figure 2.4) (Fromme et al., 2004).

There are several DNA glycosylases which have both nuclear and mitochondrial forms. Mitochondrial and nuclear forms of OGG1 are generated by alternative splicing. α-OGG1, which localizes to the nucleus and mitochondria and β-OGG1, which localizes only to mitochondria (Nishioka et al., 1999; Takao et al., 1998) α-OGG1 is likely to be the enzyme responsible for glycosylase activity in mitochondria, while the function of β-OGG1 is yet to be identified.

2.6.1. NEIL1 DNA Glycosylase

The mammalian DNA glycosylases that belong to the endonuclease VIII (Nei) family in *E. coli* have recently been identified. These enzymes are NEIL1, NEIL2 and NEIL3, which are specific for repair of oxidatively damaged bases. NEIL1 and NEIL2 are bifunctional DNA glycosylases with broad substrate specificity, no glycosylase activity has been characterized for NEIL3 so far. Among the three NEILs, N-terminal Pro, present only in NEIL1 (Figure 2.5) and NEIL2, acts as the active site nucleophile (Doublié et al., 2004).

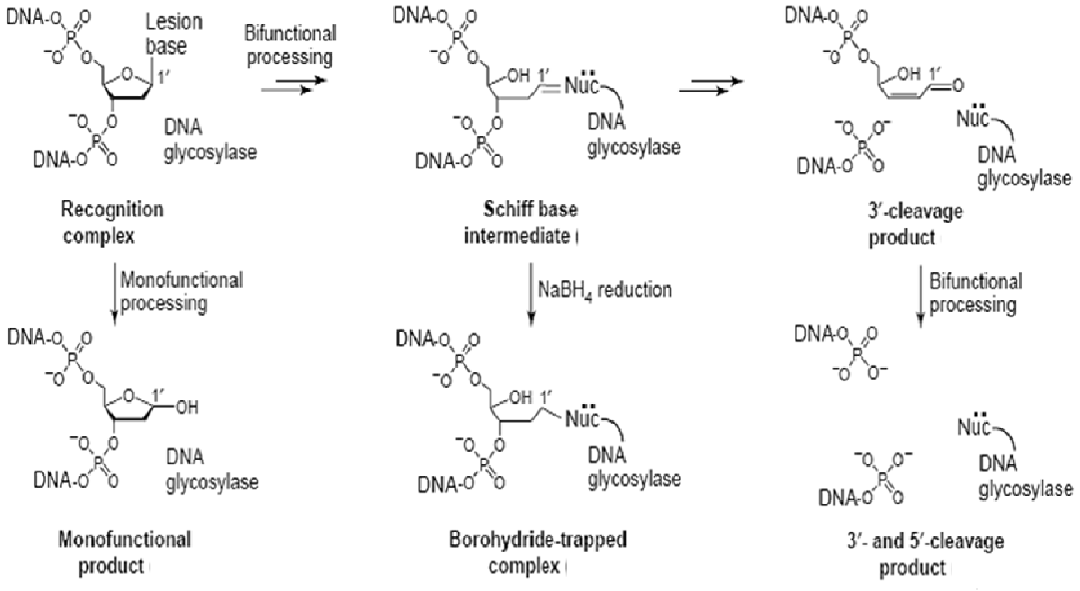

Figure 2.4. Reaction mechanisms of monofunctional and bifunctional DNA glycosylases. Formation of a borohydride-trapped enzyme-DNA complex (a Schiff Base) using $NaBH_4$ as a reducing agent (Fromme et al., 2004).

NEIL1 consists of 390 amino acids and has a molecular weight of 43 kDa. The *NEIL1* gene is mapped on chromosome 15q24.2 and spans 8,179 bp. NEIL1 is important for the repair of modified pyrimidine substrates and ring-opened purines. However 8-OH-Gua is a poor substrate for NEIL1. It has been shown that the activity of NEIL1 and its substrate specificity depend largely on the DNA structure. NEIL1 has significant 5-OH-Ura excision activity towards single stranded or bubble

DNA structures. While OGG1 and NTH1 excise the DNA damage from double strand DNA (dsDNA) structures, NEIL1 can work on single stranded DNA (ssDNA), bubble, fork and dsDNA structures. DNA bubble structures are transiently formed *in vivo* during both transcription and DNA replication, suggesting that NEIL1 might have a role in the replication and transcription-associated repair processes. Supporting this, NEIL1 levels were found to increase during the S phase of cell cycle (Dou et al., 2003; Hazra et al., 2002).

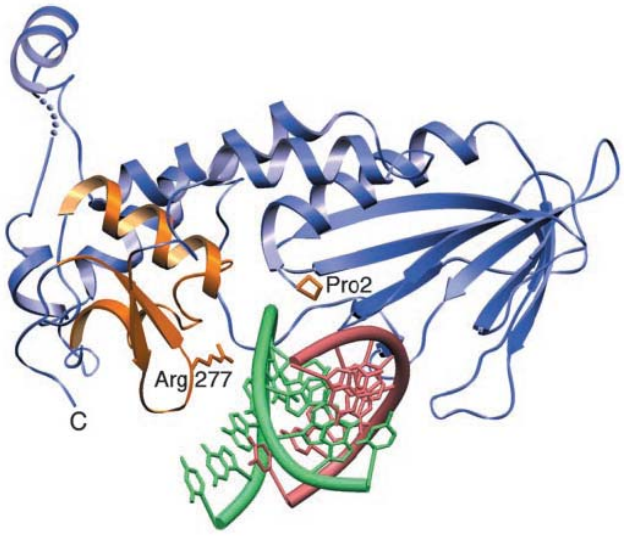

Figure 2.5. A NEIL1–DNA model. Lesion-containing strand is shown in green, complementary strand is shown in red. DNA was superimposed onto human NEIL1 which is shown in blue. The zincless finger (DNA binding motif), H2TH (DNA binding motif), catalytic proline, and arginine residue which is conserved in both *E. coli* and humans, are highlighted in gold (Doublié et al., 2004).

NEIL1 localizes both in nucleus and mitochondria, indicating that it is crucial for maintenance of both the nuclear and the mitochondrial genome (Hu et al., 2005; Morland et al., 2002). It was shown that NEIL1 mRNA levels are increased after ROS treatment (Das et al., 2005). NEIL1 deficient cells are found to be sensitive to γ-irradiation (Rosenquist et al., 2003). This indicates a critical role for NEIL1 in repairing oxidative DNA damage. Mitochondrial DNA (mtDNA) damage and deletions are increased in NEIL1 knockout ($NEIL1^{-/-}$) mice. It is demonstrated

that NEIL1 knockout ($NEIL1^{-/-}$) and NEIL1 heterozygotic mice ($NEIL1^{+/-}$) develop the majority of the symptoms of metabolic syndrome, including obesity, fatty liver disease, hyperinsulinemia and dyslipidemia (Vartanian et al., 2006). Thus NEIL1 plays an important role in the prevention of the diseases associated with metabolic syndrome. However the mechanism has not been identified yet. It has been suggested that an insufficient repair of ROS induced DNA lesions in mtDNA might be one of the reasons of the metabolic syndrome observed in $NEIL1^{-/-}$ mice. Another reason might be that because NEIL1 can initiate repair in ssDNA and bubble structures, the lack of this repair may lead to severe decreases in mitochondrial replication and transcription rates and thus an overall disruption in energy homeostasis (Vartanian et al., 2006).

3. MATERIALS AND METHOD

3.1. Materials and Chemicals

Glycerol was purchased from Invitrogen (Carlsbad, CA, USA). HEPES, Xylene Cyanole and Formamide was purchased from Sigma (St. Louis, MO, USA). Sodium Hydroxide was purchased from VWR (West Chester, PA, USA). Potassium Chloride was purchased from Baker-Analyzed Reagent (Phillipsburg, NJ, USA). 0.5 M EDTA pH 8.0 (Molecular Biology Grade) was purchased from Quality Biological Ltd. (Gaithersburg, MD, USA). Tris Buffer 1 M pH 7.4 was purchased from K.D. Medical (Columbia, MD, USA). Sodium Chloride (Molecular Biology Grade) was purchased from Quality Biological Ltd. (Gaithersburg, MD, USA). Nonidet P-40 was purchased from Bio World (Dublin, OH, USA), Albumin Standard was purchased from Pierce (Rockford, IL, USA). 12% Tris-Glycine gel was purchased from Invitrogen (Carlsbad, CA, USA). T4 polynucleotide kinase was obtained from New England Biolabs. [γ-^{32}P]ATP was from Amersham Biosciences. Ultra-pure urea and glycerol was purchased from Invitrogen (Carlsbad, CA, USA). 40% Acrylamide/Bis Solution 19:1 was purchased from Bio-Rad (Hercules, CA, USA). Typhoon trio + Variable Mode PhosphorImager from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Vacufuge Concentrator 5301 from Eppendorf (Enfield, CT, USA).

3.2. Recombinant Proteins

Recombinant N-terminal hemaglutinin antigen (HA)- and C-terminal histidine$_6$ (His)-double-tagged human CSB protein was purified from HiFive insect cells (Christiansen et al., 2003). *E. coli* formamidopyrimidine DNA glycosylase (Fpg) was purified as previously described in Reddy et al., in 2004. Recombinant Fpg and CSB proteins were purified in the Laboratory of Molecular Gerontology, National Institute on Aging, National Institutes of Health, USA. Purified recombinant NEIL1 protein was kindly provided by Dr. Sankar Mitra (University of Texas Medical Branch, Galveston, USA).

3.3. Oligodeoxynucleotides

Synthetic single lesion oligodeoxynucleotide constructs have been instrumental for the understanding of repair pathways for modified DNA bases. The sequences of the oligodeoxynucleotides used in this study are shown in Table 3.1 FapyGua-containing oligodeoxynucleotide was a kind gift from Dr. Marc Greenberg (Jiang et al., 2005). The oligodeoxynucleotides containing 5-OH-Ura or 8-OH-Gua were obtained from Midland Certified Reagent Co. (Midland, TX, USA).

Table 3.1. The sequence of DNA oligonucleotides. **F**: FapyGua or **U:** 5-OH-Ura or **G:** 8-OH-Gua.

Oligodeoxynucleotides	**Sequences**
FapyGua	5'- CCAGGTGC**F**AAGTGGT-3' 5'-AGCACGTCCCAT-3' 3'-GGTCCACGCTTCACCATCGTGCAGGGTA-5'
Duplex 5-OH-Ura	5'-GCTTAGCTTGGAATCGTATCATGTA**U**ACTCGTGTGCCGTGTAGACCGTGCC-3' 3'-CGAATCGAACCTTAGCATAGTACATGTGAGCACACGGCACATCTGGCACGG-5'
Bubble 5-OH-Ura	5'-GCTTAGCTTGGAATCGTATCATGTA**U**ACTCGTGTGCCGTGTAGACCGTGCC-3' 3'- CGAATCGAACCTTAGCATAGGCACCCGACAAACACGGCACATCTGGCACGG-5'
8-OH-Gua	5'-GCTTAGCTTGGAATCGTATCATGTA**G**ACTCGTGTGCCGTGTAGACCGTGCC-3' 3'-CGAATCGAACCTTAGCATAGTACATCTGAGCACACGGCACATCTGGCACGG-5'

3.4. Construction of radiolabeled DNA substrate containing FapyGua

3.4.1. Radiolabeling the oligodeoxynucleotides with ^{32}P

Oligodeoxynucleotide A (Table 3.1, blue color) was radiolabeled at its 5' end by kinase reaction (See Table 3.2).

Table 3.2. Reaction mixture used for labelling of oligodeoxynucleotide A.

Kinase reaction A	**Oligodeoxynucleotide A 5'-CCAGGTGCFAAGTGGT-3'**	**Final concentration**
Oligodeoxynucleotide A (10 pmol/µl)	7.5 µl	2.5 pmol/µl
T4 PNK (20 U)	3 µl	2 Unit
10X TBE	3 µl	1X
[γ-^{32}P]ATP (10 µCi)	4.5 µl	1.5 µCi
dH_2O	12 µl	

Oligonucleotide B (Table 3.1, red color) was phosphorylated using ATP at its 5` end by kinase reaction (See Table 3.3).

Table 3.3. Reaction mixture used for phosphorylation of oligodeoxynucleotide B.

Kinase reaction B	**Oligodeoxynucleotide B 5'-AGCACGTCCCAT-3'**	**Final concentration**
Oligodeoxynucleotide A (10 pmol/µl)	5.2 µl	4 pmol/µl
T4 PNK (20 U)	2.6 µl	2 Unit
10 X TBE	2.6 µl	1X
10 mM ATP	1.3 µl	0.5 mM
dH_2O	14.3 µl	

After the addition T4 PNK to the kinase reactions of oligodeoxynucleotides A and B, the reaction mixtures (A and B) were incubated at 37°C for 1 hour. The kinase reaction was inactivated by heating at 65°C for 20 minutes. To remove the unincorporated [γ-^{32}P]ATP and ATP, the reaction mixtures were loaded onto a Sephadex G-25 column and centrifuged at 800xg for 2.5 minutes, and the eluate was collected for the annealing reaction.

3.4.2. Ligation of oligodeoxynucleotides A and B, and purification of radiolabeled 28mer FapyGua oligodeoxynucleotide

The kinase reaction mixtures A (30 µl) and B (26 µl) were combined in a 1mL tube in a total volume of 56 µl. To this mixture, 4.5 µl of template (4 pmol/µl; 3'-GGTCCACGCTTCACCATCGTGCAGGGTA-5') (Table 3.1), 7 µl of 10X ligase buffer (400mM Tris-HCl pH 7.8, 100mM $MgCl_2$, 100mM DTT) and 2.5 µl water were added (total volume 70 µl). Tubes were incubated at 80°C for 10 minutes, and then cooled down to 30°C to facilitate double strand formation. The reaction mixture was incubated with 9 µl of 10 mM ATP, 11 µl of T4 DNA ligase in a total volume of 90 µl at 16°C for 1.5 hours for ligation. After the incubation, denaturing loading dye (95 % formamide, 20 mM EDTA pH 8.0, 0.02% bromophenol blue and 0.02% xylene cyanol) was added and the samples were incubated for 4 minutes at 80°C. This DNA mixture was loaded onto a 20% 19:1 acrylamide/*bis*-acrylamide/7M urea denaturing gel to separate the radiolabeled ligated 28mer FapyGua-containing oligodeoxynucleotides from enzyme, radiolabeled 16mer oligodeoxynucleotides, truncated pieces of DNA or misannealed oligodeoxynucleotides. Gel was exposed to X-ray film and the desired radiolabeled FapyGua-containing oligodeoxynucleotides were excised from the gel. The gel pieces were cut into very small pieces and eluted with 400 µl of elution buffer (1 mM EDTA pH 8.0, 100 mM NaCl) at 37°C for 7 hours. Reaction mixture was loaded onto a Sephadex G-25 column and centrifuged for 10 minutes at 16,100xg. The DNA (FapyGua-containing oligodeoxynucleotides) was precipitated in the presence of 0.2 volume of ammonium acetate and 2.5 volume absolute ethanol at -20°C overnight. The supernatant was removed after 1 hour of centrifugation at 20,187xg. The DNA precipitate was washed twice with 70% cold ethanol to remove the salts. The DNA samples were then dried in a vacufuge

concentrator and DNA was re-suspended in 20 µl 10 mM Tris, pH 8.0. Radiation emission was measured on a Scintillation counter.

3.4.3. Annealing reaction

For the annealing reaction, 36 µl of dH_2O, 2 µl of 1 M LiCl, and 20 pmol (2 µl, 10 pmol/µl) of complementary oligodeoxynucleotide (Table 3.1) were added to the tube containing 10µl of ^{32}P labeled FapyGua-containing oligodeoxynucleotides (see subheading 3.4.2). The mixture was incubated at 95°C for 5 minutes and then the heat block was turned off and oligodeoxynucleotides were allowed to anneal while cooling to room temperature over a period of 4-5 hours. The annealed DNA substrates containing FapyGua were stored at 4°C.

3.5. Construction of radiolabeled DNA substrates containing 5-OH-Ura or 8-OH-Gua

3.5.1. Radiolabeling the oligodeoxynucleotide with ^{32}P

The gel purified oligodeoxynucleotides containing 5-OH-Ura or 8-OH-Gua (Table 3.1) were radiolabeled at their 5'-end by kinase, reaction mixture is seen in Table 3.4.

Table 3.4. Reaction mixture used for phosphorylation of 51 mer oligodeoxynucleotide with 5-OH-Ura lesion.

Kinase reaction	Amounts	Final Concentration
Oligodeoxynucleotide	1 µl	10 pmol/µl
T4 PNK (20 U)	1 µl	2 U
10X T4 PNK	1 µl	1X
[γ-^{32}P]ATP (10 µCi)	1 µl	1 µCi
dH_2O	6 µl	

After the addition of 1 µl of T4 PNK, the reaction mixture was incubated at 37°C for 1 hour. The kinase reaction was inactivated by heating at 65°C for 20 minutes. To remove the unincorporated [γ-^{32}P]ATP, 10 µl of the reaction mixture was loaded onto a Sephadex G-25 column and centrifuged at 700 g for 1.5 minutes, and the eluate was collected.

3.5.2. Annealing reaction

For the annealing reaction, 36 µl of dH_2O, 2 µl of 1 M LiCl, and 20 pmol (2 µl, 10 pmol/µl) of complementary oligonucleotides (Table 3.1) were added to the kinase reaction mixture, final volume 10 µl (see subheading 3.5.1). The mixture was incubated at 95°C for 5 minutes and then the heat block was turned off and allowed the oligonucleotides to anneal upon cooling to room temperature over a period of 4-5 hours. The annealed DNA substrates were stored at 4ºC.

3.6. DNA glycosylase activity assays for NEIL1

3.6.1. Incision assay (DNA glycosylase+AP lyase activities)

NEIL1 is a class II DNA glycosylase, which catalyzes nucleophilic cleavage of the N-glycosyl bond, and subsequently converts the resulting abasic site to a single strand DNA break via AP lyase activity (McCullough et al. 1999). To measure both DNA glycosylase and AP lyase activities of NEIL1, the incision experiment were performed. Incision of FapyGua, 5-OH-Ura or 8-OH-Gua (see subheadings 3.4 and 3.5) was performed in a reaction mixture (10 µl) containing 40 mM HEPES-KOH, pH 7.6, 1 mM EDTA, 100 mM KCl, 2 mg/µl bovine serum albumin (BSA), 10% glycerol, and 5 fmol/µl of each ^{32}P-labeled DNA substrate. The incision reactions were initiated by adding 2.5 fmol/µl NEIL1 and increasing concentrations of CSB (2.5, 6.25, 12.5 fmol/µl). The reactions were incubated for 30 minutes at 37 °C and terminated by adding 2X formamide stop dye (95% formamide, 20 mM EDTA pH 8.0, 0.02% bromophenol blue, 0.02% xylene cyanol, 10% SDS and 5 µg/µl proteinase K), followed by 15 minutes incubation at 37°C. Samples were heated at 95°C for 5 minutes and then ran on 20% 19:1 acrylamide/*bis*-acrylamide/7 M urea denaturing gel at 15 W for 2 hours in 1X TBE buffer (89mM Tris base; 89 mM Boric acid; 2mM EDTA pH 8.0). Gels were

exposed to phosphorImager screen overnight and visualized by PhosphorImager (Typhoon Trio). Results were quantitated with ImageQuant 5.2 software. The percentage of incision was calculated by the amount of radioactivity present in the product band relative to the total radioactivity.

3.6.2. DNA glycosylase activity assay

To measure NEIL1 DNA glycosylase activity, NaOH was used in the incision assay described in subheading 3.6.1 to chemically cleave all the AP sites generated by NEIL1 (Marsin et al., 2003). This assay was conducted in the absence or presence of 100 mM NaOH. In a DNA glycosylase assay (10 µl total reaction volume), 5 fmol/µl of FapyGua substrate was incubated in glycosylase reaction buffer (40 mM HEPES-KOH, pH 7.6, 1mM EDTA, 100 mM KCl, 2 mg/µl bovine serum albumin (BSA), and 10% glycerol) with 2.5 fmol/µl NEIL1 and increasing concentrations of CSB (2.5, 6.25, 12.5 fmol/µl). Reactions were carried out at 37°C for 30 minutes. After this step, equivalent aliquots of these reaction mixtures (4 µl) were analyzed with addition of 2X formamide stop dye (see subheading 3.6.1) with 100 mM NaOH or without NaOH. Then the samples were incubated for 15 minutes at 37°C. After the incubation, samples were heated at 95°C for 5 minutes and then ran on 20% 19:1 acrylamide/*bis*-acrylamide/7 M urea denaturing gel at 15 W for 2 hours in 1X TBE buffer. Gels were exposed to PhosphorImager screen overnight and visualized by PhosphorImager (Typhoon Trio). Reactions were quantitated with ImageQuant 5.2 software. The percentage of incision was calculated using the amount of radioactivity present in the product band relative to the total radioactivity.

3.7. AP lyase activity assay

The AP lyase activity of NEIL1 involves a β,δ-elimination reaction (Figure 2.4) and a transient Schiff base intermediate between the enzyme and the DNA substrate. In the presence of reducing agent sodium borohydride ($NaBH_4$), NEIL1 becomes irreversibly linked to its DNA substrates (Figure 2.4). The enzyme-DNA complex which naturally occurs transiently is hence “trapped”. Once covalently bound, trapped molecules no longer participate in the overall reaction resulting in stable glycosylase–DNA complexes. DNA trapping assays were performed as

described for the glycosylase assay in subheading 3.6.1, with the addition of freshly prepared 50 mM $NaBH_4$ at the start of the reactions. After incubation at 37°C for 2 hours, the reactions were terminated by adding 5X SDS-PAGE sample buffer, and the samples were heated at 95°C for 5 minutes. Trapped protein-DNA complexes were separated in 12% SDS-PAGE. The gels were visualized using PhosphorImager, and analyzed using the ImageQuant software (Amersham Biosciences).

4. RESULTS

4.1. CSB stimulates incision activity of NEIL1

The activities of NEIL1 in the presence or absence of recombinant CSB were examined, using oligodeoxynucleotide substrates containing different DNA lesions or structures including FapyGua, 5-OH-Ura or 8-OH-Gua (Table 2.1).

4.1.1. CSB stimulates NEIL1 incision activity on FapyGua substrate

NEIL1 efficiently incised a 28mer oligodeoxynucleotide at the single FapyGua site, generating the expected 9mer product (Figure 4.1, lane 3). A constant amount of NEIL1 (2.5 fmol/µl) was incubated in the presence of increasing concentrations of recombinant CSB for 30 minutes at 37°C. The addition of increasing concentrations of recombinant CSB (2.5, 6.25, 12.5 fmol/µl), from 1:1 to 1:2.5 molar ratio, linearly increased NEIL1 incision activity in a concentration-dependent fashion, by approximately 4-fold compared to NEIL1 alone (Figure 4.1, compare lanes 4-5, and Figure 4.2). The addition of higher amounts of CSB (1:5 molar ratio) further increased NEIL1 incision only slightly, probably indicating a saturation of the interaction (Figure 4.1, lane 6 and Figure 4.2). The ability of CSB to stimulate NEIL1 incision activity was heat labile (Figure 4.1, lane 7). Furthermore, CSB alone did not incise the substrate (Figure 4.1, lane 2), indicated that CSB did not have an inherent incision activity and CSB protein preparation was free of any contaminants that might cause DNA incision activity. These results indicate that CSB stimulates NEIL1 incision activity on a FapyGua-containing DNA substrate.

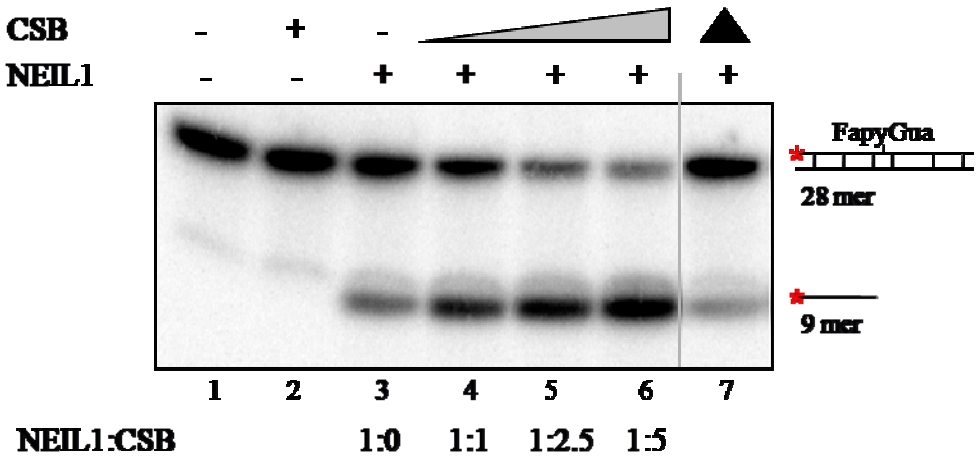

Figure 4.1. Stimulation of NEIL1 incision activity on FapyGua-containing substrate by CSB. NEIL1 (2.5 fmol/µl) was incubated in the presence or absence of increasing concentrations of CSB (0, 2.5, 6.25, 12.5 fmol/µl ;lanes 3-6 respectively) with 5'-^{32}P-labeled FapyGua-containing substrate (5 fmol/µl) for 30 minutes at 37°C. Reaction products were ran on a 20% denaturing polyacrylamide gel and visualized by a PhosphorImager. Lane 1, substrate alone. Lane 2, CSB (12.5 fmol/µl) alone. Lane 7, ▲, 12.5 fmol/µl heat-denatured CSB protein.

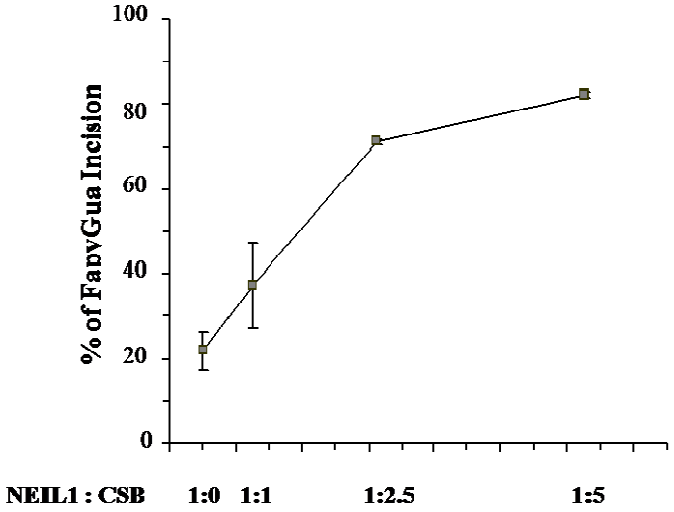

Figure 4.2. Percentage of FapyGua incision from three independent experiments is plotted. The percentage of incision is calculated as the amount of radioactivity present in the product band relative to the total radioactivity. Bars, SD.

4.1.2. CSB stimulates NEIL1 incision activity on 5-OH-Ura substrate

For 5-OH-Ura is also a substrate for NEIL1 (Dou et al., 2003), the effect of CSB on NEIL1 incision activity on a duplex or bubble substrates containing 5-OH-Ura was examined. CSB stimulated NEIL1 incision activity on duplex substrate in a dose dependent manner (Figure 4.3, lanes 4-6 and Figure 4.5) but not on bubble substrate containing 5-OH-Ura (Figure 4.4, lanes 4-6 and Figure 4.5). Heat-inactivated CSB did not alter NEIL1 activity (Figure 4.3, lane 7), indicating that the stimulation of CSB to the activity of NEIL1 on 5-OH-Ura containing duplex substrate was heat labile.

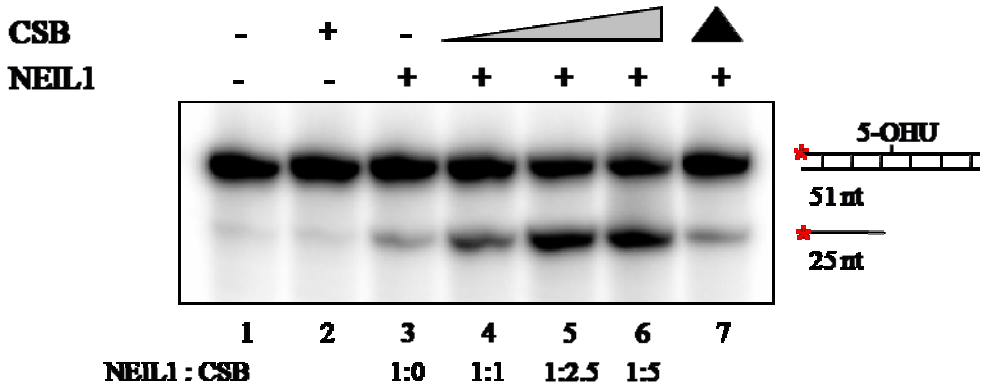

Figure 4.3. CSB affects NEIL1 incision activity on 5-OH-Ura containing duplex substrate. NEIL1 (2.5 fmol/µl) was incubated in the presence or absence of increasing amounts of CSB (0, 2.5, 6.25, 12.5 fmol/µl ; lanes 3-6 respectively) with 5'-^{32}P-labeled 5-OH-Ura-containing duplex substrate (5 fmol/µl) for 30 minutes at 37°C. Reaction products were run on a 20% denaturing polyacrylamide gel and visualized by a PhosphorImager. Lane 1, substrate alone. Lane 2, CSB (12.5 fmol/µl) alone. Lane 7, ▲, 12.5 fmol/µl heat-denatured CSB protein.

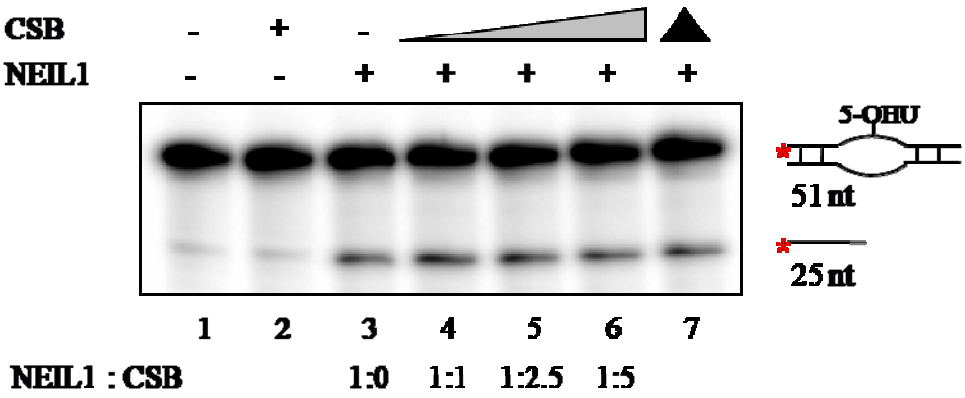

Figure 4.4. CSB does not affect NEIL1 incision activity on 5-OH-Ura bubble substrate. NEIL1 (2.5 fmol/µl) was incubated in the presence or absence of increasing amounts of CSB (0, 2.5, 6.25, 12.5fmol/µl; lanes 3-6 respectively) with 5'-^{32}P-labeled 5-OH-Ura-containing bubble substrate (50 fmol/µl) for 30 minutes at 37 °C. Lane 1, substrate alone. Lane 2, CSB (12.5 fmol/µl) alone. Lane 7, ▲, 12.5 fmol/µl heat-denatured CSB protein.

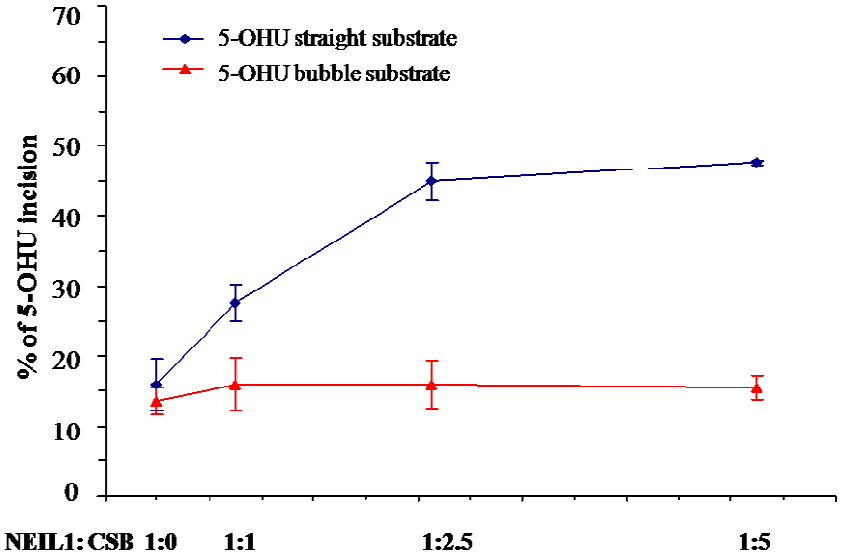

Figure 4.5. Percentage of 5-OH-Ura incision from three independent experiments is plotted. Bars, SD.

4.1.3. CSB does not stimulate NEIL1 incision activity on 8-OH-Gua substrate

To obtain comparable incision activity as to that observed with the other two lesions (see subheadings 4.1.1 and 4.1.2), four times the concentration of NEIL1 (10 fmol/µl) was used in the assays (Fig. 4.3, lane 2). Moreover, addition of increasing concentrations of recombinant CSB, up to 50 fmol/µl (1:5 molar ratio) did not affect NEIL1 incision of the 8-OH-Gua–containing substrate (Fig. 4.3, lanes 3-6). Thus, 8-OH-Gua is not a major substrate for NEIL1, and recombinant CSB does not enhance NEIL1 incision activity on 8-OH-Gua-containing substrate (Figure 4.6, lanes 3-6).

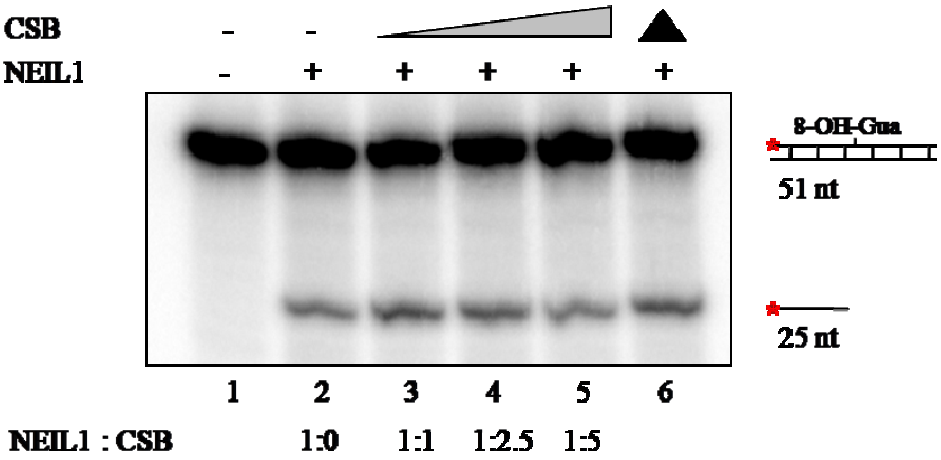

Figure 4.6. CSB does not affect NEIL1 incision activity on 8-OH-Gua substrate. NEIL1 (10 fmol/µl) was incubated in the presence or absence of increasing amounts of CSB (0, 10, 25, 50 fmol/µl ; lanes 2-5 respectively) with 8-OH-Gua-containing substrate (5 fmol/µl) for 30 minutes at 37°C. Lane 1, substrate alone.

4.1.4. CSB does not stimulate incision activity of bacterial *E. coli* formamidopyrimidine DNA glycosylase (Fpg)

To determine whether CSB stimulation of incision activity was specific for the NEIL1 protein, the effect of CSB on Fpg was tested. Fpg is a bacterial homologue of NEIL1, which recognizes a similar spectrum of DNA lesions. At 0.5 fmol/µl of Fpg protein, increasing CSB concentrations (0.5-5 fmol/µl) had no

detectable effect on Fpg activity on 5-OH-Ura containing substrate (Figure 4.7, lanes 3-7 and Figure 4.8). In contrast, reactions done in the same experiment has shown that CSB stimulated NEIL1 incision activity (Figure 4.7, lanes 8-9). These results attest to the specificity of the CSB stimulation of incision activity for NEIL1.

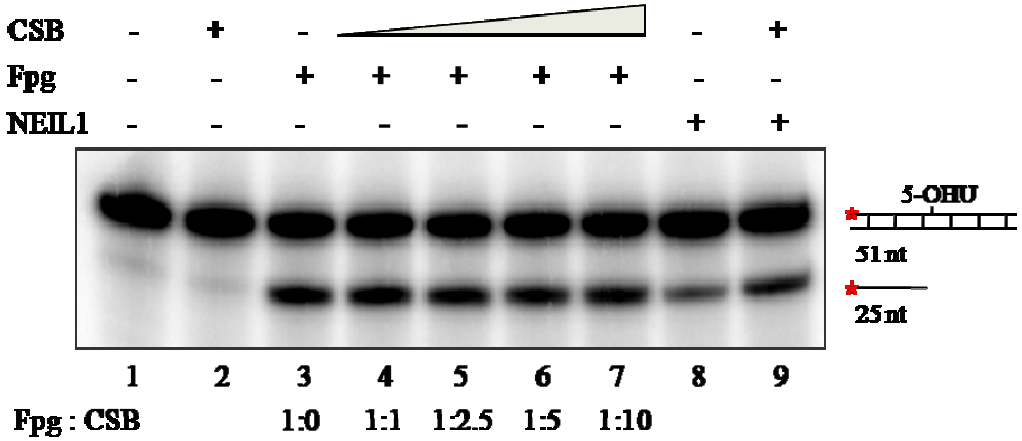

Figure 4.7. Fpg incision activity. Fpg (0.5 fmol/µl) was incubated with increasing CSB amounts (0, 0.5, 1.25, 2.5, 5 fmol/µl ; lanes 3-7), and NEIL1 (2.5 fmol/µl ; lane 8) was incubated with 2.5 fmol/µl CSB (lane 9) for 30 minutes at 37°C. Lane 1, substrate alone. Lane 2, CSB (5 fmol/µl) alone.

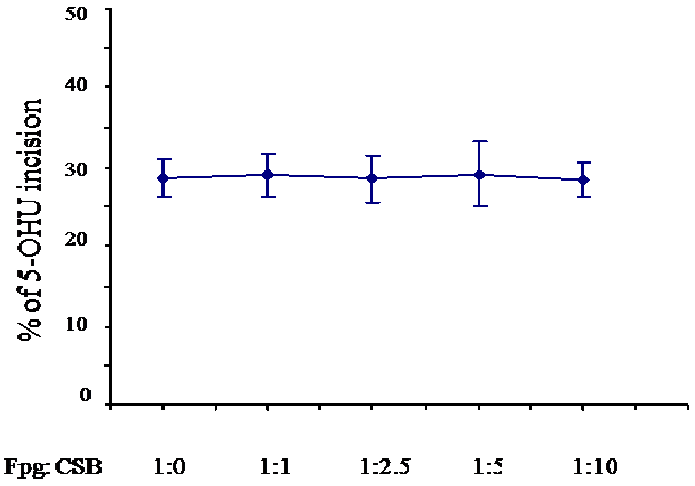

Figure 4.8. Percentage of 5-OH-Ura incision from three independent experiments is plotted. Bars, SD.

4.2. CSB stimulates the AP-lyase activity of NEIL1

Class II DNA glycosylases such as NEIL1 catalyze two reactions. The first is a nucleophilic attack that targets the N-glycosyl bond and releases the damaged base. The enzyme then attacks the abasic sugar, ultimately leading to a single strand break (McCullough et al., 1999). This AP-lyase activity, which can be carried out via β,δ-elimination reactions, forms a transient Schiff base intermediate with the substrate. In the case of some DNA glycosylases such as OGG1, these activities are uncoupled (Zharkov et al., 2000). Whether NEIL1 DNA glycosylase and AP-lyase activities were equally stimulated by CSB was investigated. There was not any significant difference in CSB stimulation of NEIL1 incision activity in presence or absence of NaOH, which cleaves remaining abasic sites (Figure 4.9), suggesting that CSB did not preferentially stimulate one activity over the other, thus uncoupling the enzyme.

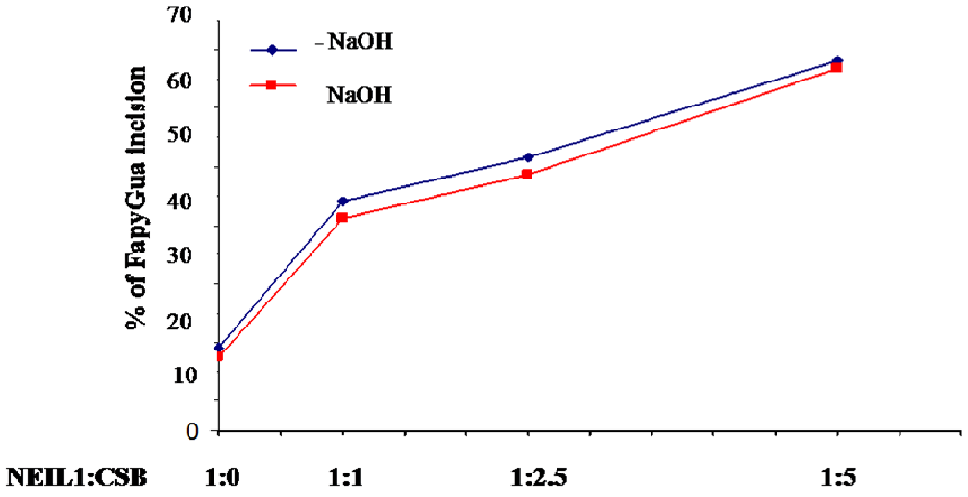

Figure 4.9. Glycosylase activity of NEIL1. NEIL1 (2.5 fmol/µl) was incubated with varying concentrations of CSB (0, 2.5, 6.25, 12.5 fmol/µl) with FapyGua–containing substrate (5 fmol/µl) at 37°C for 30 minutes. The reactions were then incubated for 5 minutes at 90°C in the presence or absence of 100 mM NaOH and resolved as described in subheading 3.6.2. The average of two independent experiments is plotted.

Then, whether CSB directly stimulated the AP-lyase activity of NEIL1 was tested. The enzyme-substrate complex formed during strand cleavage can be converted into a covalently "trapped complex" by reduction with a strong reducing agent, such as sodium borohydride (Sun et al., 1995). Using this approach, the addition of CSB, at 1:1 molar ratio, to the reaction resulted in a three-fold increase of the $NaBH_4$-trapped NEIL1-FapyGua-substrate (Figure 4.10, lane 4 and Figure 4.11). This stimulation is also dose-dependent (Figure 4.10, lanes 4-6 and Figure 4.11), resulting in almost 8-fold increase in the trapped complex with a 1:5 NEIL1:CSB molar ratio. Since the Schiff base is formed after or simultaneously with the excision of the base, these results suggest that CSB stimulates either the binding of NEIL1 to the damaged DNA or the incision step itself rather than the enzyme turnover.

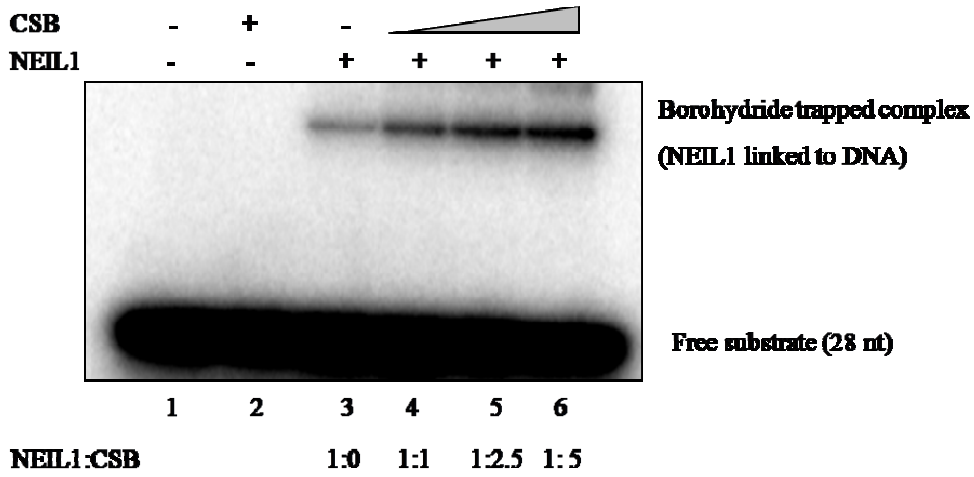

Figure 4.10. CSB stimulates the AP lyase activity of NEIL1 on FapyGua substrate. NEIL1 (2.5 fmol/µl) was incubated with varying concentrations of CSB (0, 2.5, 6.25, 12.5 fmol/µl ; lanes 3-6 respectively) with FapyGua-containing substrate (5 fmol/µl) in the presence of 50 mM $NaBH_4$ at 37°C for 30 minutes and analyzed by 12% SDS-PAGE. Lane 1, substrate alone. Lane 2, CSB (12.5 fmol/µl) alone.

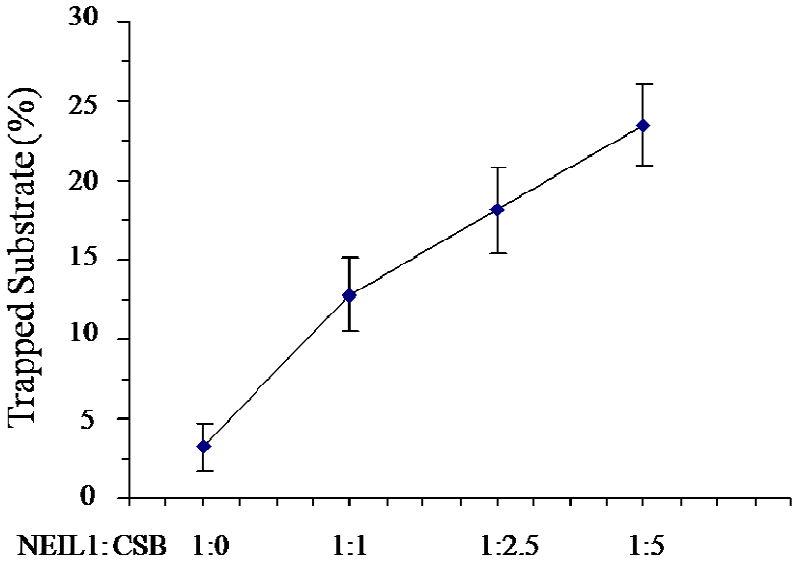

Figure 4.11. Percentage of trapped substrate from three independent experiments is plotted. Bars, SD.

5. DISCUSSION

UV sensitivity is a hallmark of cells from CS patients, and CSB protein is required for TCR of UV-induced DNA lesions [CPD and 6-pyrimidine-4-pyrimidone products] (van Oosterwijk et al., 1996). However, these biochemical and cellular properties do not directly explain the progressive neurodegeneration observed in the CS patients. Since UV light can not reach the brain, and the neurons are subjected to high levels of oxidative stress due to their high oxidative metabolism, it has been proposed that deficiency in oxidative DNA damage repair could be responsible for the neurological symptoms seen in CS patients (Kraemer et al., 2007).

The involvement of the CSB protein in BER of oxidative DNA damage has been suggested by the hypersensitivity of CSB deficient cells to oxidative stress (de Waard et al., 2004), accumulation of oxidative DNA lesions, and increased genomic instability in CSB deficient models (Stevnsner et al., 2008). Accumulation of oxidative DNA lesions have been directly associated with aging, carcinogenesis and neurodegenerative diseases (de Souza-Pinto et al., 2002; Loft and Poulsen, 1996; Mattson and Liu, 2002). It has been shown that 8-OH-Gua and 8-OH-Ade accumulate in primary and SV-40 transformed CSB deficient fibroblasts (Tuo et al., 2001; 2002). In addition, it has recently been demonstrated that FapyGua and FapyAde oxidative lesions significantly accumulate in brain, liver and kidney DNA of CSB knockout mice that have not been exposed to exogenous sources of oxidative stress (Muftuoglu et al., unpublished data). These findings suggest that CSB may play a direct role in the repair of these lesions, and elevated levels of FapyGua and FapyAde may play a role in the pathophysiology of CS.

The substrate specificities of DNA glycosylases are broad and, in some cases, overlapping (Ide and Kotera, 2004). FapyGua, FapyAde and 5-OH-Ura are major substrates for NEIL1 DNA glycosylase. While 8-OH-Gua has been considered a substrate for NEIL1, it is not excised by NEIL1 from DNA containing multiple lesions. 8-OH-Gua and FapyGua are also substrates for OGG1 DNA glycosylase (Hegde et al., 2008). Overlapping substrate specificities may explain the absence of obvious phenotypes in knockout mice lacking single DNA glycosylases

such as OGG1 and the endonuclease III homologue (NTH1) knockout mice. However, knockout mice lacking NEIL1 develop a combination of clinical manifistations resembling human metabolic syndrome as it is observed in CS patients (Vartanian et al., 2006). This observation suggests that *in vivo* NEIL1 preferentially repairs lesions that have severe biological consequences, and these lesions are not entirely removed by other DNA glycosylases (Vartanian et al., 2006).

Since FapyGua and FapyAde accumulate in CSB knockout mice tissues, this thesis hypothesizes that CSB may play a role in the repair of formamidopyrimidines together with NEIL1 DNA glycosylase. In this study, it was found that CSB stimulates NEIL1 incision activity in a lesion-specific manner. A concentration dependent stimulation was observed with FapyGua and 5-OH-Ura–containing substrates, but not with 8-OH-Gua–containing substrate. These results are in line with the observation that 8-OH-Gua is not a relevant substrate for NEIL1 when the DNA substrate contains multiple lesions. Furthermore, *E. coli* Fpg, a sequence and functional homologue of NEIL1, was not stimulated by CSB, even at high protein concentrations. Thus, the stimulating effect of CSB is specific for NEIL1.

The stimulation of NEIL1 activity at low CSB molar ratios is also very significant, which is approximately 4-fold by 1:2.5 molar ratio (Figure 4.1). Recently, Dou et al. showed that PCNA interacts with and stimulates NEIL1 incision activity (Dou et al., 2008). However they report, with the best substrate, a 3-fold stimulation with a ~1:83.3 molar ratio of NEIL1:PCNA (Dou et al., 2008). Likewise, Guan and colleagues report a stimulation of NEIL1 by the checkpoint sensor complex 9-1-1; they observed a 3.8-fold stimulation with a 1:50 molar ratio of NEIL1:9-1-1 (Guan et al., 2007).

Unlike most DNA glycosylases, NEIL1 acts on lesions positioned in single-stranded DNA, particularly in a single-stranded bubble in a duplex sequence (Dou et al., 2003). This feature has led to the hypothesis that NEIL1 functions in the repair of oxidative damage in the context of transcription. CSB is also known to bind single-stranded DNA and is part of the RNA polymerase II elongating complex (Sarker et al., 2005). Interestingly, CSB did not stimulate NEIL1 incision of a substrate containing the oxidative lesion in a bubble structure (Figure 4.4). CSB

binds stalled elongating RNA polymerase II complexes (containing bubble structures) (Iyer et al., 1996) and its DNA-dependent ATPase activity is highest with DNA substrates containing small loops or 15-nucleotide bubbles (Christiansen et al., 2003). In this context, it is possible that higher affinity/interaction of CSB protein with such secondary structures in the substrate would cancel off its stimulation of NEIL1 incision activity. This result suggests that the CSB/NEIL1 functional interaction may be of greater relevance in general genome BER.

In this sudy, the molecular mechanism by which CSB stimulated NEIL1 incision activity was also investigated. It was found that CSB increases the strand cleavage step. The NEIL1-catalyzed incision of a FapyGua-containing substrate was stimulated by CSB both in the absence as well as in the presence of NaOH, indicating that the base release step is not uncoupled from the strand cleavage step (Figure 4.9)**.** On the other hand, the amount of sodium borohydride-trapped DNA-NEIL1 complexes increased in the presence of CSB (Figures 4.10 and 4.11), indicating an enhanced strand cleavage activity. Although these reactions were not performed under single turnover conditions (25 fmol/μL of enzyme and 50 fmol/μL of DNA substrate), it is likely that the interaction with CSB alters kinetic properties of NEIL1.

It has recently been demonstrated that CSB also physically interacts with NEIL1, as the two proteins can be co-precipitated from HeLa nuclear extracts and co-localize in the nuclei of intact cells, as demonstrated by immunocytochemistry (Muftuoglu et al., unpublished data). CSB stimulation of NEIL1 incision was protein concentration dependent, with significant increase in activity with a molar ratio of 1:1 (Figure 4.1). This indicates that this protein interaction may have a significant impact on NEIL1 activity *in vivo*. Previous studies suggest that CSB redistributes in cells exposed to H_2O_2, and that this redistribution requires *c*-Abl-induced phosphorylation of CSB (Imam et al., 2007). Thus, CSB may be recruited to oxidative DNA lesions, where the NEIL1/CSB interaction is functionally relevant.

It is possible that CSB plays an important role in the repair of oxidatively modified bases via its interaction with lesion-specific DNA glycosylases. This study presents the first functional interaction of CSB with NEIL1 DNA glycosylase to

repair FapyAde, FapyGua and 5-OH-Ura oxidative DNA lesions. In addition, CSB physically interacts with NEIL1. Although CSB affects the function of OGG1 in the repair of 8-OH-Gua, there is no direct physical interaction between CSB and OGG1, however; the proteins were shown to be present in the same protein complex (Dianov et al., 1999; Tuo et al., 2002). In addition to NEIL1 and OGG1, two other DNA glycosylase activities, thymine glycol DNA glycosylase (hNTH) and uracil DNA glycosylase (hUDG), have been monitored in CSB deficient cells. hNTH is specific for the incision of 5-hydroxycytosine and hUDG for the incision of uracil lesion. It was shown that CSB does not affect the incision activities of these DNA glycosylases (Dianov et al., 1999).

In summary, CSB and NEIL1 interaction possibly plays an important role in repair of endogenous and induced formamidopyrimidine *in vivo*. Moreover, these results clearly demonstrate a role for CSB in general genome BER of oxidatively induced lesions and further studies are required to establish how CSB is involved in the regulation of BER.

6. CONCLUSION

In the present study, the functional cooperation between CSB and NEIL1 was investigated. CSB greatly enhances NEIL1 incision activity on various DNA substrates, via a stimulation of the strand incision step. The results of incision assays for NEIL1 with 28 nucleotide FapyGua containing substrate were found as follows: with 1:1 molar ratio of NEIL1 and CSB, the amount of incised substrates was increased by 2-fold compared to NEIL1 alone. The addition of 1:2.5 molar ratio linearly increased NEIL1 incision activity in a concentration-dependent fashion, by approximately 4-fold compared to NEIL1 alone. The addition of higher amounts of CSB (1:5 molar ratio) further increased NEIL1 incision only slightly, probably indicating a saturation of the interaction.

CSB stimulated NEIL1 incision activity on the duplex 5-OH-Ura substrate in a dose dependent manner, but not on the bubble substrate containing 5-OH-Ura. Heat-inactivated CSB and CSB alone did not modulate or incise the NEIL1 activity, respectively on both FapyGua and 5-OH-Ura substrates.

NEIL1 incision assays performed with 51 nucleotide 8-OHGua substrate demonstrated that the stimulating effect of CSB on NEIL1 incision activity was specific to FapyGua and 5-OH-Ura. Since, increasing CSB concentrations had no effect on the incision activity of NEIL1 on 8-OH-Gua substrate. In addition, at least 4 times more NEIL1 (100 fmol/μL versus 25 fmol/μL in the assays with FapyGua and 5-OH-Ura) was necessary to obtain comparable incision activity as to that observed with the other two lesions.

The specificity of the NEIL1 stimulation by CSB using another enzyme that cleaves 5-OH-Ura–containing substrates, *E. coli* Fpg was tested. Addition of CSB did not stimulate cleavage at the 5-OH-Ura site. On the other hand, NEIL1 activity increased with CSB addition under the same experimental conditions. Thus, CSB stimulation is specific to NEIL1, and that its bacterial homologue, which recognizes a similar spectrum of lesions, is not stimulated by CSB.

Since NEIL1 is a bifunctional DNA glycosylase, whether NEIL1 DNA glycosylase and AP-lyase activities were equally stimulated by CSB was examined. CSB did not stimulate the NEIL1 incision activity in presence or absence of NaOH,

which cleaves remaining abasic sites, suggesting that CSB did not preferentially stimulate one activity over the other, thus uncoupling the enzyme. The addition of CSB, at 1:1 molar ratio resulted in a three-fold increase of the $NaBH_4$-trapped NEIL1-FapyGua substrate, indicating that CSB directly stimulates the AP-lyase activity of NEIL1.

7. REFERENCES

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