Cockayne Syndrome Group B Protein Stimulates Repair of Formamidopyrimidines by NEIL1 DNA Glycosylase

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Cockayne syndrome (CS) is a segmental premature aging syndrome with progressive neurological degeneration (1). CS is caused by mutations in CS complementation groups A (CSA) or B (CSB) genes (2, 3). Approximately 80% of CS patients have mutations in the CSB gene, which encodes a 168-kDa protein belonging to the SWI/SNF2 family of chromatin remodeling proteins (4). Cells from CS patients are hypersensitive to UV radiation-induced DNA damage, and the CSB protein is required for the transcription-coupled nucleotide excision repair of UV radiation-induced DNA lesions (5). CSB is also believed to play a role in transcription elongation and interacts with the RNA polymerase II elongation complex (6). The molecular basis of the progressive neurological defects in CS patients, however, remains unknown; 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 (7).

Oxidative DNA damage can be caused by endogenous and exogenous agents. Reactive oxygen species, including highly reactive hydroxyl radicals, are formed as byproducts of normal metabolism, mostly during the process of mitochondrial respiration. It has been estimated that up to 2% of all the O₂ consumed by respiration may be released as reactive oxygen species (8, 9). The central nervous system relies exclusively on mitochondria to generate ATP through oxidative metabolism. As a result, neurons are susceptible to increased levels of oxidative stress, and elevated levels of reactive oxygen species have been implicated in the etiology of neurodegenerative diseases including Alzheimer, Parkinson, and Huntington diseases and amyotrophic lateral sclerosis (for a review, see Ref. 10).

Hydroxyl radicals attack DNA bases and the sugar-phosphate DNA backbone, generating modified bases and single-stranded DNA (ssDNA) breaks, respectively (11). Many oxidatively damaged sites are recognized and repaired by the base excision repair (BER) pathway, which includes the DNA glycosylases and AP endonuclease pathways (12). 8-OH-Gua and FapyGua are two such oxidatively induced DNA lesions that are known to be repaired by BER enzymes (13). 8-OH-Gua and FapyGua have been shown to accumulate in the brain and kidney tissues of CSB-deficient mice (14, 15). In addition, CSB stimulates AP endonuclease activity in vitro, and CSB and NEIL1 co-immunoprecipitate and co-localize in HeLa cells (7). When CSB and NEIL1 are depleted from HeLa cells by short hairpin RNA knockdown, repair of induced FapyGua is strongly inhibited. This result suggests that CSB plays a role in repair of formamidopyrimidines, possibly by interacting with and stimulating NEIL1, and that accumulation of such modifications may have a causal role in the pathogenesis of CS.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

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The abbreviations used are: CS, cockayne syndrome; BER, base excision repair; OGG1, 8-oxoguanine DNA glycosylase; 8-OH-Gua, 7,8-dihydro-8-oxoguanine; PARP1, poly(ADP-ribos)e polymerase; Fpg, formamidopyrimidine DNA glycosylase; shRNA, short hairpin RNA; GC, gas chromatography; MS, mass spectrometry; LC, liquid chromatography; 5-OH-Ura, 5-hydroxycytosine; GC, gas chromatography; 5-OH-Gua, 5-hydroxoguanine; ssDNA, single-stranded DNA; Fapy, formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-Fapy; FapyAde, 4,6-diamino-5-Fapy; PBS, phosphate-buffered saline; wt, wild type; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; CSBfI, full-length CSB; AP, apurinic or apyrimidinic.

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tively induced DNA lesions are mutagenic and/or cytotoxic and have been associated with aging, neurodegeneration, and carcinogenesis (for review, see Refs. 12 and 13). Most of these lesions are repaired by the base excision repair pathway (BER), during which lesion-specific DNA glycosylases hydrolyze the N-glycosidic bond between the modified base and the sugar moiety to release the modified base and generate an abasic (AP) site. Subsequent repair steps include cleavage of the resulting abasic site, incorporation of one or few nucleotides, trimming of the 5’ and 3’ ends, and ligation of the DNA backbone (14).

Several DNA glycosylases specifically recognize and repair oxidatively induced DNA lesions (15). The substrate specificities of DNA glycosylases are broad and, in some cases, overlapping, which may explain the absence of severe phenotypes in knock-out mice lacking 8-oxoguanine DNA glycosylase (OGG1) or endonuclease III homologue (NTH1). In contrast, mice lacking endonuclease VIII-like (NEIL1) DNA glycosylase have a combination of clinical manifestations resembling human metabolic syndrome (16). This phenotype suggests that NEIL1 is essential for repair of an endogenous DNA lesion or lesions that has significant biological consequences when incorrectly or incompletely repaired.

Recent evidence suggests that CSB may play a role in repair of oxidatively induced DNA damage. Embryonic fibroblasts from csb<sup>−/−</sup> mice are hypersensitive to γ-irradiation and paraquat, a redox-cycling compound that generates oxidative stress (17), and γ-irradiated human cells expressing mutated CSB have defects in repair of 7,8-dihydro-8-oxoguanine (8-OH-Gua) and 7,8-dihydro-8-oxoadenine (18, 19). Furthermore, CSB is found of CSB and NEIL1 were chosen for further analysis. For the tested for knockdown levels of CSB and NEIL1 by Western blot analysis. The clones demonstrating the lower expression levels of CSB and NEIL1 were chosen for further analysis. For the treatments the clones were plated into 15-cm dishes (in triplicate) and incubated until 80–90% confluence was reached. The cells were exposed to 200 μM menadione (in PBS) for 30 min at 37 °C. At the end of the treatment the drug was removed, the cultures were washed twice with PBS, and the cells were immediately harvested for DNA isolation, or the culture medium was replaced, and the cells were incubated for 6 h at 37 °C for repair. After the repair period, the cultures were washed once with PBS, and the cells were harvested for DNA isolation.

Preparation of Mouse Brain, Liver, and Kidney Mitochondrial or Nuclear Extracts—CSB knock-out mice (csb<sup>−/−</sup>), kindly provided by Dr. Jan Hoeijmakers (Erasmus University Medical Center, Rotterdam, Netherlands), were bred at the Gerontology Research Center Animal Facility under standard conditions. Wild type (wt) litters were used as controls. Mice were sacrificed by cervical dislocation, and the brain, liver, and kidney were immediately removed and processed. Nuclear and mitochondrial extracts were prepared as described earlier (33). All experiments were approved by the Gerontology Research Center Animal Care and Use Committee and performed in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, National Institutes of Health Publication 85-23, National Institutes of Health, Bethesda, MD.

Preparation of mtDNA and nDNA from Mouse Brain, Liver, and Kidney—DNA was isolated using a modification of the salting-out method (34). Mitochondrial or nuclear pellets from brain, liver, and kidney were suspended in 2 ml of buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM Hepes-KOH, pH 7.4, 2 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol, 0.15 mM extracts. Short hairpin RNAs (shRNA) double knockdown of CSB and NEIL1 greatly inhibits repair of FapyGua. These results suggest that NEIL1 and CSB cooperate in the repair of FapyAde and FapyGua.

EXPERIMENTAL PROCEDURES

Recombinant Proteins—Recombinant N-terminal hemagglutinin antigen- and C-terminal His<sub>6</sub>-double-tagged human CSB protein was purified from HiFive insect cells as described previously (31). Escherichia coli formamidopyrimidine DNA glycosylase (Fpg) was isolated and purified as described (32). Purified recombinant NEIL1 protein was kindly provided by Dr. Sankar Mitra (University of Texas Medical Branch, Galveston, TX).

Cell Lines and shRNA Transfection—HeLa cells were maintained at 37 °C, 5% CO<sub>2</sub> in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). For knockdown experiments, the cells were transfected with 50 nM shRNA targeting either human CSB (OriGene, catalog no. TR313176), human NEIL1 (OriGene, catalog no. TR307276), or non-targeting control shRNA (OriGene) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Stable NEIL1 KD and CSB/NEIL1 double KD cells were selected and maintained in 2 μg/ml puromycin. Individual clones were selected and tested for knockdown levels of CSB and NEIL1 by Western blot analysis. The clones demonstrating the lower expression levels of CSB and NEIL1 were chosen for further analysis. For the treatments the clones were plated into 15-cm dishes (in triplicate) and incubated until 80–90% confluence was reached. The cells were exposed to 200 μM menadione (in PBS) for 30 min at 37 °C. At the end of the treatment the drug was removed, the cultures were washed twice with PBS, and the cells were immediately harvested for DNA isolation, or the culture medium was replaced, and the cells were incubated for 6 h at 37 °C for repair. After the repair period, the cultures were washed once with PBS, and the cells were harvested for DNA isolation.

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spermine, and 0.75 mM spermidine. The homogenates were then diluted in lysis buffer containing 10 mM Tris-HCl, pH 8.2, 2 mM EDTA, 400 mM NaCl, 1% SDS, to an approximate protein concentration of 1 mg/ml. Two mg/ml of proteinase K (Sigma) were added, and the samples were incubated at 37 °C overnight. One-fourth volume of saturated NaCl was added, and proteins were precipitated by centrifugation at 16,100 × g for 15 min. The supernatant was recovered, and the DNA was precipitated with 2.5 volumes of 96% ethanol at −20 °C for 1–2 h. The precipitated DNA was collected by centrifugation, suspended in 5 ml of 10 mM Tris, 1 mM EDTA, and incubated with RNase A (0.1 mg/ml) at 37 °C for 1 h. The samples were then subjected to another proteinase K (0.5 mg/ml) digestion in lysis buffer at 55 °C for 1 h. After precipitation of proteins with saturated NaCl and centrifugations as above, DNA was precipitated from the supernatant with ethanol, collected by centrifugation, and dried in a SpeedVac.

Analysis by Gas Chromatography/Mass Spectrometry (GC/MS) and Liquid Chromatography LC/MS—All DNA samples were blinded before measurement. DNA samples were dissolved in water. The DNA quality and concentration in each sample were determined by the UV spectrum recorded between 200 and 350 nm. The identification and quantification of 8-OH-Gua, FapyGua, and FapyAde in DNA was performed by GC/MS after hydrolysis of DNA samples by E. coli Fpg (32, 35). 50-μg aliquots of DNA samples were supplemented with aliquots of 8-OH-Gua-[13C2,15N2], [13C,15N]FapyAde, and [13C,15N]FapyGua as internal standards and then hydrolyzed with 2 μg of Fpg as described (32). After ethanol precipitation, DNA pellets and supernatant fractions were separated by centrifugation. Supernatant fractions were lyophilized, trimethylsilylated, and analyzed by GC/MS as described (32). For identification and quantification, selected-ion monitoring was used to monitor the characteristic ions of trimethylsilylated 8-OH-Gua, FapyAde, and FapyGua and their stable isotope-labeled analogues as internal standards (36).

LC/MS was used to identify and quantify 8-OH-Gua as its nucleoside, 8-OH-dG, in DNA samples. 50-μg aliquots of DNA samples were supplemented with an aliquot of 8-OH-[13C2,15N2]dG as internal standard. Samples were hydrolyzed with nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase for 24 h at 37 °C and then analyzed by LC/MS as described (27). For identification and quantification, selected-ion monitoring was used to monitor the characteristic ions of 8-OH-dG and its internal standard (37). The levels of 8-OH-Gua measured with GC/MS and LC/MS were essentially identical (supplemental Fig. S1). Statistical analyses of GC/MS and LC/MS results were performed using a Kruskal-Wallis test with S-Plus 7 statistical software. A p value equal to or lower than 0.05 was considered to be statistically significant.

Oligodeoxynucleotides—The sequences of the oligodeoxynucleotides used here are shown in Table 1. FapyGua-containing oligodeoxynucleotides were a kind gift from Dr. Marc Greenberg (38). The oligodeoxynucleotides containing 5-OH-Ura or 8-OH-Gua were obtained from Midland Certified Reagent Co. (Midland, TX). All oligodeoxynucleotides were 5’-end-labeled using T4 polynucleotide kinase and [γ-32P]ATP as described before (39). FapyGua-, 8-OH-Gua-, and 5-OH-Ura-containing oligodeoxynucleotides were annealed to the complementary strand in 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 100 mM KCl by heating the samples at 90 °C for 5 min and slowly cooling to room temperature.

DNA Glycosylase Assays for NEIL1—Incision of FapyGua, FapyAde, 5-OH-Ura, or 8-OH-Gua was performed in a reaction mixture (10 μl) containing 40 mM Hepes-KOH, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 50 mM NaCl, 0.2 μg/μl bovine serum albumin, 5% glycerol, and 50 fmol of [32P]-labeled DNA substrate, except for FapyAde, which contained 5 fmol. The reactions were initiated by adding NEIL1 and CSB as indicated in the figure legends 2–5. The reactions were incubated at 37 °C for 30 or 60 min and stopped with the addition of 5 μg of proteinase K and 1 μl of 10% SDS followed by 15 min of incubation at 37 °C. To promote complete strand cleavage at the abasic sites, 100 mM NaOH was added and incubated at 37 °C for 15 min. An equal amount of formamide loading dye (90% formamide, 10 mM EDTA, 0.01% bromphenol blue, 0.01% xylene cyanol) was added, and the samples were incubated at 95 °C for 5 min and resolved by electrophoresis on a 20% polyacrylamide, 7 mA urea gel. Gels were visualized by PhosphorImager and analyzed using the ImageQuant software (GE Healthcare). The percentage of incision was calculated as the amount of radioactive activity present in the product band relative to the total radioactive activity. To measure NEIL1 AP-lyase activity, the experiments were processed as described above without incubation with NaOH.

DNA Trapping Assay—DNA trapping assays were performed as described for the glycosylase assay, with the addition of freshly made 50 mM NaBH4 at the start of the reactions. After incubation at 37 °C for 2 h, the reactions were terminated by adding 5 μl of 5X SDS-PAGE sample buffer, and the samples were heated at 95 °C for 5 min. Trapped-protein-DNA complexes were separated in 12% SDS-PAGE. The gels were visualized using PhosphorImager and analyzed using the ImageQuant software (GE Healthcare).

Construction of Recombinant CSB Fragments and CSB Point Mutant Proteins—CSB-(2–341) and CSB-(514–986) fragments were purified as previously described (24). Briefly, the fragments were cloned into the pTriEx-4 Neo vector (Novagen), which encodes N-terminal His and S tags and C-terminal herpes simplex virus and His tags. The two fragments were overexpressed in E. coli and purified using the His and S tags. Construction of pcDNA3.1-CSB646Q and CSBQ942E plasmids, making stable transfected CS1AN cell lines and purification of CSB wild type and mutant proteins, were done as described previously (31, 40, 41).

Co-immunoprecipitation Assay—HeLa nuclear extracts were prepared as described previously (42) and were precleared for 1 h with rProtein G-agarose beads (Invitrogen). Extracts (250 μg each) were incubated with either 4 μg of rabbit anti-CSB antibody (Santa Cruz Biotechnology), 4 μg of rabbit anti-NEIL1 antibody (Calbiochem), or 4 μg of rabbit IgG (Santa Cruz Biotechnology) as a negative control for 16 h at 4 °C. Each sample was then incubated with rProtein G-agarose beads (30 μl) at 4 °C for 1 h. Bound proteins were eluted by boiling in SDS sample buffer for 5 min and analyzed by Western blotting with goat anti-CSB (1:1000; Santa Cruz Biotechnology) or goat anti-
NEIL1 (1:500; Santa Cruz Biotechnology) antibodies for 16 h at 4 °C followed by chemiluminescent analysis (Pierce).

**Immunofluorescence Staining**—HeLa cells were plated on four-well Lab-Tek Chamber Slides and grown for 24 h. Cells were washed 3 times with PBS and fixed in freshly prepared 4% paraformaldehyde in PBS for 15 min at room temperature. The cells were then washed 3 times in PBS and permeabilized in cold 0.25% Triton X-100 solution in PBS for 10 min on ice followed by three washes in PBS. Cells were blocked for unspecific protein binding using 1% bovine serum albumin in PBS for 30 min at room temperature. After blocking, primary antibodies diluted in washing buffer (0.5% bovine serum albumin and 0.1% Tween 20 in PBS) were added and incubated overnight at 4 °C. After 3 washes in washing buffer, fluorescent-labeled secondary antibody diluted in washing buffer was added and incubated for 45 min at room temperature. Slides were washed at least six times in washing buffer, any remaining washing buffer was removed, and cells were mounted with hard set mounting media containing 4',6-diamidino-2-phenylindole (Vector-Shield). Primary antibodies used were CSB H-300 rabbit (diluted 1:500) and NEIL1 S-17 goat (diluted 1:75) (Santa Cruz); secondary antibodies were Alexa Fluor 488 donkey anti-goat and Alexa Fluor 594 donkey anti-rabbit. Antibodies were checked for cross-reaction and bleed-through between the two channels by using one primary and both secondary antibodies. No cross-reaction or bleed-through was detected. Background from the secondary was checked using no primary and both secondary. Pictures were acquired on a Nikon Eclipse TE-2000e confocal microscope using the 60X objective lens, 0.2-μm z-stacks throughout the cells with the Volocity software. Quantification was done using Volocity software, selecting the more intense areas and only including anything bigger than one voxel as follows; signals with intensity four or more S.D. above the mean intensity were selected for both NEIL1 and CSB. Anything with a volume less than 1.5 voxel for both NEIL1 and CSB were excluded. Co-localization was measured as overlap between the selected CSB and NEIL1 signals that was bigger than 1.5 voxels. An average of three experiments (total of 90 cells counted) is presented together with representative pictures. For the images, low level intensity signal was removed, and the contrast was enhanced in Volocity software.

**RESULTS**

**Elevated Levels of FapyGua, FapyAde, and 8-OH-Gua in DNA from CSB Knock-out Mouse Tissues**—It has been proposed that oxidative DNA damage accumulates in CSB-deficient cells and that this plays a role in the pathophysiology of CS, especially in the dysfunction of the central nervous system. However, previous studies either measured 8-OH-Gua specifically or sites sensitive to *E. coli* Fpg, which recognizes 8-OH-Gua, FapyGua, and FapyAde collectively (35). Here, the endogenous levels of 8-OH-Gua, FapyGua, and FapyAde were measured in liver, brain, and kidney genomic DNA and in liver mtDNA from wt or csb−/− mice by GC/MS. The results show that 8-OH-Gua is present at a significantly higher level in genomic DNA from brain and kidney of csb−/− than wt mice but not in liver DNA (Fig. 1A). FapyGua levels are also significantly higher in brain and kidney from csb−/− mice (Fig. 1B). FapyAde levels, on the other hand, are ~2-fold higher in all three organs of csb−/− mice (Fig. 1C). The level of the nucleoside counterpart of 8-OH-Gua, 8-OH-dG, was measured in liver nuclear DNA using LC/MS, and almost identical results were obtained with GC/MS, demonstrating that both techniques are comparably suitable to measure this lesion (supplemental Fig. S1).

Previous studies also suggest a role for CSB in repairing oxidative DNA damage in mitochondria (43). In the current study analysis of liver mtDNA showed that FapyAde levels are higher in csb−/− mice than in wt mice but that the levels of FapyGua and 8-OH-Gua were similar (Fig. 1D).

**CSB Stimulates Incision Activity of NEIL1**—The results presented above suggest that FapyAde accumulates in multiple tissues in csb−/− mice. FapyGua and 8-OH-Gua, but not FapyAde, are substrates for OGG1 (44). However, both FapyAde and FapyGua are substrates for NEIL1 (27–29). This suggests that NEIL1 activity may be compromised in mice lacking CSB and that CSB might cooperate with NEIL1 in recognizing and processing FapyGua. This idea was tested by carrying out NEIL1 incision assays in vitro in the presence or absence of recombinant CSB using synthetic oligodeoxynucleotide substrates containing FapyGua, FapyAde, 5-hydroxyuracil (5-OH-Ura), or 8-OH-Gua (Table 1). NEIL1 efficiently incised a 28-mer oligode-
CSB Stimulates NEIL1 DNA Glycosylase

oxygenucleotide containing a single FapyGua, generating the predicted 9-mer product (Fig. 2A, lane 3). The addition of increasing amounts of recombinant CSB (up to 2.5-fold molar excess) stimulated NEIL1 incision activity up to 4-fold (Fig. 2A, compare lanes 3 and 5, and Fig. 2B). However, higher amounts of CSB (at 5-fold molar excess) further increased NEIL1 incision activity only slightly, indicating a saturation of the interaction (Fig. 2A, lane 6). Similar results were obtained with an oligodeoxynucleotide containing a FapyAde (Fig. 2C). The incubation of the 30-mer FapyAde-containing oligodeoxynucleotide with NEIL1 generated the expected 15-mer incision product. Recombinant CSB stimulated NEIL1 FapyAde incision activity in a dose-dependent manner up to 2-fold in a 2.5 molar ratio (Fig. 2D). Dou et al. (45) recently showed that proliferating cell nuclear antigen also interacts with and stimulates NEIL1 incision activity. However, in that study proliferating cell nuclear antigen stimulated NEIL1 incision activity up to 3-fold at a much higher molar excess of proliferating cell nuclear antigen (∼1.83:1). Likewise, Guan et al. (46) reported a stimulation of NEIL1 by the checkpoint sensor complex 9-1-1; they observe a 3.8-fold stimulation with a 1:50 molar ratio of NEIL1:9-1-1. Control reactions show that heat inactivation of CSB eliminates its ability to stimulate NEIL1 incision activity (Fig. 2A, lane 7) and that CSB alone does not directly incise FapyGua (Fig. 2A, lane 2). This indicates that CSB does not have an inherent glycosylase activity nor does the CSB preparation contain any contaminating DNA glycosylase activity. These results indicate that CSB stimulates NEIL1-catalyzed incision activity of FapyGua in vitro at an equimolar level.

Specificity of the CSB-NEIL1 Interaction—In contrast to most DNA glycosylases, NEIL1 is active on DNA lesions in ssDNA, particularly in the context of a single-stranded bubble in a duplex sequence (47). Because of this property, it has been suggested that NEIL1 plays a role during transcription. CSB also binds ssDNA and is part of the RNA polymerase II elongating

TABLE 1

| Oligodeoxynucleotides used for incision assays | Fg, FapyGua; Fa, FapyAde; 5'-OH-Ura, 5'-OH-Gua, 8-OH-Gua. The unpaired region in the bubble-containing oligo is underlined. | Fg, FapyGua; Fa, FapyAde; 5'-OH-Ura, 5'-OH-Gua, 8-OH-Gua. The unpaired region in the bubble-containing oligo is underlined. |
|-----------------------------------------------|------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| Oligodeoxynucleotides and sequences | | |
| FapyGua | 5'-CCAGTCCACGCCTGCTGCTCCTGGGTA-5' | 5'-CCAGTCCACGCCTGCTGCTCCTGGGTA-5' |
| FapyAde | 5'-CCTTCAACCTCTGTACATGGAAGTTGCACGTGAT-5' | 5'-CCTTCAACCTCTGTACATGGAAGTTGCACGTGAT-5' |
| Duplex 5'-OH-Ura | 5'-GCTTAGCTTGGAATCGTATCATGTA-3' | 5'-GCTTAGCTTGGAATCGTATCATGTA-3' |
| Bubble 5'-OH-Ura | 5'-CTTCAACGTGCACTGAGAACCTTAGCATAGTGACCCCGACAAACACGGCACATCTGGCACGG-5' | 5'-CTTCAACGTGCACTGAGAACCTTAGCATAGTGACCCCGACAAACACGGCACATCTGGCACGG-5' |
| 8-OH-Gua | 5'-GCTTAGCTTGGAATCGTATCATGTA-3' | 5'-GCTTAGCTTGGAATCGTATCATGTA-3' |

FIGURE 2. Stimulation of NEIL1 glycosylase activity on FapyGua- and FapyAde-containing substrate by CSB. A, NEIL1 (25 fmol) was incubated in the presence or absence of increasing amounts of CSB (0, 25, 62.5, 125 fmol; lanes 3–6, respectively) with 5'-32P-labeled FapyGua-containing substrate (50 fmol) for 1 h 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 (125 fmol) alone. A, 125 fmol (lane 7) of heat-denatured CSB protein. B, quantification of percentage of FapyGua incision from three independent experiments is plotted. The percentage of incision is calculated as the amount of radioactive present in the product band relative to the total radioactivity. C, NEIL1 (10 fmol) was incubated in the presence or absence of increasing amounts of CSB (0, 10, 20, 25 fmol; lanes 2–5, respectively) with 5'-32P-labeled FapyAde-containing substrate (5 fmol) for 30 min at 37 °C. Reaction products were run on a 20% denaturing polyacrylamide gel and visualized by a PhosphorImager. Lane 1, substrate alone; lane 6, CSB (25 fmol) alone. D, quantification of percentage of FapyAde incision from three independent experiments is plotted. The percentage of incision is calculated as the amount of radioactive present in the product band relative to the total radioactivity.

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Thus, the ability of CSB to stimulate NEIL1 activity was examined using a bubble or a duplex DNA substrate containing a 5-OH-Ura. Both substrates contain the same sequence context in the damage-containing strand. Interestingly, CSB stimulated NEIL1 incision activity on the duplex substrate in a dose-dependent manner (Fig. 3, A, lanes 4–6, and C) but not on the bubble substrate containing 5-OH-Ura (Fig. 3, B, lanes 4–6, and C). CSB stimulation of NEIL1-catalyzed incision of this DNA substrate was also heat-labile (Fig. 3, A, lane 7).

NEIL1 excises 8-OH-Gua very inefficiently (27–29). Here we observed that at least 4-fold more NEIL1 (100 versus 25 fmol) was required to incise a similar fraction of single-lesion DNA substrates containing 8-OH-Gua as FapyGua or 5-OH-Ura (Fig. 3D, lane 2). In addition, up to a 5-fold molar excess of recombinant CSB did not stimulate incision of 8-OH-Gua by NEIL1 (Fig. 3D, lanes 3–6). The specificity of the interaction between NEIL1 and CSB was examined by testing whether CSB stimulates the ability of E. coli Fpg to incise 5-OH-Ura. The results show that CSB does not stimulate Fpg-catalyzed incision of 5-OH-Ura in dsDNA (supplemental Fig. S2A, lanes 4–7, and S2B). In contrast, reactions done in the same experiment showed that CSB stimulated NEIL1 incision activity (supplemental Fig. S2A, lanes 8 and 9). These results suggest that CSB stimulation is specific to NEIL1, and that its bacterial homologue, which recognizes a similar spectrum of lesions, is not stimulated by CSB.

**CSB Stimulates the AP Lyase Activity of NEIL1**—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 ssDNA break via AP lyase activity (49). The AP lyase activity of NEIL1 involves a β,δ-elimination reaction and a transient Schiff base intermediate between the enzyme and the DNA substrate. For some DNA glycosylases, including OGG1, these activities are uncoupled (50). Here, we investigated whether CSB stimulates NEIL1 DNA glycosylase and AP lyase activities equally. The amount of CSB/NEIL1 incision product was unchanged by the addition of NaOH (supplemental Fig. S3), which cleaves the remaining abasic sites, suggesting that CSB did not preferentially stimulate one activity over the other. However, the addition of sodium borohydride to trap NEIL1 with the FapyGua-containing substrate revealed that equimolar CSB stimulated the NEIL1-mediated AP lyase activity 3-fold (Fig. 4A, lane 4, and B). This stimulation is also dose-dependent (Fig. 4A, A, lanes 4–6, and B), resulting in an almost 8-fold increase in the trapped complex with a 1:5 NEIL1:CSB molar ratio. Because the Schiff base is formed after or at the same time as excision of the base, these results suggest that CSB stimulates...
either binding to or cleavage of DNA lesions by NEIL1 instead of stimulating enzyme turnover.

The N-terminal Part and Not the ATPase Domain of CSB Stimulate NEIL1 Incision Activity—CSB has been shown to interact with PARP1 in vivo and in vitro (24). PARP1 is known to bind ssDNA breaks and to be involved in BER. The interaction between CSB and PARP1 has been mapped to the N terminus of CSB, more specifically to CSB-(2–341). Furthermore, CSB has been shown to be poly(ADP-ribo)sylated in vitro after oxidative stress, and the most dominant site of poly(ADP-ribo)sylation in vitro has been shown to be the same N-terminal fragment (24), indicating a role for this domain of CSB in BER. The ATPase activity of CSB has been shown to be crucial for its role in the recovery of cells from UV sensitivity, RNA synthesis recovery, and apoptosis (40, 41). We, therefore, investigated whether the N-terminal fragment CSB-(2–341) or the CSB-(514–986) fragment containing the ATPase domain are involved in the stimulation of NEIL1 incision activity (Fig. 5B). CSB fragments CSB-(2–341) and CSB-(514–986) were overexpressed and purified, and the purity of full-length CSB (CSBfl), CSB-(2–341), and CSB-(514–986) was assessed using Coomassie stain (Fig. 5A, lanes 1–3). 5-OH-Ura containing duplex substrate was incubated with NEIL1 with or without CSBfl, CSB-(2–341), or CSB-(514–986). NEIL1 incision activity was stimulated by CSBfl and CSB-(2–341) in a 2.5 molar ratio of CSB to NEIL1 (Fig. 5B, compare lane 2 to lanes 3 and 4). CSB-(514–986), in the same molar ratio, did not stimulate NEIL1 incision activity (compare lane 2 to lane 5). The extent of NEIL1 stimulation was similar using CSBfl or CSB-(2–341) and around 3-fold (Fig. 5, B and C). Hence, the N-terminal part of CSB stimulates NEIL1 incision activity to the same extent as CSBfl, whereas the ATPase domain fragment does not stimulate NEIL1 incision activity.

Whole cell and mitochondrial extracts from CSB-deficient cells have been shown to exhibit decreased 8-OH-Gua incision activity when compared with extracts from cells complemented with wild type CSB. Interestingly, extracts from the cells expressing the CSBE646Q mutation in the ATPase motif II, abolishing the ATPase activity, are still able to complement the decreased 8-OH-Gua incision (31, 40, 41, 43), indicating that the ATPase activity is not needed for the function of CSB in BER. Thus, we tested the effect of single point mutations in the CSB protein, CSBE646Q (domain II) and CSBQ942E (domain VI), on its stimulation of NEIL1 incision activity. The CSBQ942E mutant affects the repair of 8-OH-Gua, indicating that CSB domain VI is important for the role of CSB in the processing of 8-OH-Gua lesions (18). These recombinant proteins were purified from insect cells, and their purity is shown in supplemental Fig. S4B. The ATPase mutant protein, CSBE646Q, showed almost the same degree of stimulation as the CSBwt protein on NEIL1 incision activity.

FIGURE 4. Molecular mechanism of CSB stimulation of NEIL1. A, NEIL1 (25 fmol) was incubated with varying concentrations of CSB (0, 25, 62.5, 125 fmol) with FapyGua-containing substrate (50 fmol) at 37 °C for 30 min in the presence of 50 mM NaBH4, and analyzed by 12% SDS-PAGE. Lane 1, substrate alone. Lane 2, CSB (125 fmol) alone. B, quantification of percentage of trapped substrate from three independent experiments is plotted; bars, S.D.

FIGURE 5. Stimulation of NEIL1 incision activity by N-terminal of CSB but not by the ATPase domain. A, purity of CSB full-length (CSBfl) and CSB fragments. To access the purity of the used CSB and CSB fragments, 400 ng of purified CSB full-length, CSB-(2–341), and CSB-(514–986) was separated on a 4–12% Bis-Tris gel by SDS-PAGE and stained using the Coomassie-based Imperial Protein Stain (ThermoScientific). B, top panel, a schematic diagram of CSB fragments. Lower panel, NEIL1 (50 fmol) was incubated in the absence or presence of CSBfl (125 fmol), CSB-(2–341) (125 fmol), or CSB-(514–986) (125 fmol) with 5’-32P-labeled 5-OH-Ura-containing duplex substrate (10 fmol) for 2 h at 37 °C. Reaction products were separated on a 20% denaturing polyacrylamide gel and visualized by a PhosphorImager. C, the percentage of incision was calculated as the amount of radioactivity present in the product band relative to the radioactivity in the substrate and product band. Shown is a representative gel from repeated experiments.
activity (supplemental Fig. S4A). This result is compatible with the result obtained with the CSB-(514–986) fragment containing the ATPase domain (Fig. 5B). In addition, the domain VI mutant, CSBQ942E, stimulated the incision activity of NEIL1 to the same extent as the CSBwt protein (supplemental Fig. S4A).

**CSB Is in Complex with NEIL1**—The results presented above demonstrate a functional interaction between CSB and NEIL1 during repair of oxidatively induced DNA lesions. We, thus, tested whether CSB and NEIL1 are found in the same complex in vivo by co-immunoprecipitation and immunohistochemical staining of CSB and NEIL1 in HeLa cells. The results showed co-immunoprecipitation of endogenous CSB (Fig. 6A, upper panel, lane 3) and NEIL1 (Fig. 6A, bottom panel, lane 3) by anti-CSB antibodies. This result was confirmed by reciprocal co-immunoprecipitation using antibody to NEIL1 (Fig. 6B, lower panel, lane 3). Control experiments showed that control IgG did not immunoprecipitate CSB or NEIL1 (Fig. 6, A, upper panel, lane 2, and B, upper panel, lane 1). To rule out the possibility that this interaction was mediated by DNA, the immunoprecipitation experiments and HeLa nuclear extract preparation were performed in the presence of DNase I. In addition, confocal microscopy of fixed permeabilized HeLa cells revealed ~25% co-localization of CSB and NEIL1 in the nucleoplasm (Fig. 6C) and some NEIL1 staining in putative mitochondrial cytoplasmic sites, as demonstrated previously (30).

**Accumulation of FapyGua in Human Cells Depleted of NEIL1 and CSB**—The possible role of NEIL1 and CSB in repair of Fapys in vivo was also examined in cells depleted of NEIL1 and CSB by shRNA. Efficient shRNA knockdown of CSB and NEIL1 was confirmed by Western blot (Fig. 7A) and knockdown, and control cells were treated with menadione to increase levels of Fapys. DNA was isolated from treated cells with or without a recovery period, and FapyGua levels were measured. Cells transfected with a control shRNA (negative control) showed a transient 3-fold increase in the level of FapyGua after treatment with menadione; in these cells induced FapyGua was completely repaired within a 6-h recovery period (Fig. 7B, first set of bars). In contrast, knockdown cells lacking NEIL1 (Fig. 7B, second set) or both CSB and NEIL1 (Fig. 7B, third set) failed to repair menadione-induced FapyGua during a 6-h recovery period. These results demonstrate that repair of induced FapyGua requires NEIL1 in HeLa cells. However, in this system shRNA knockdown of NEIL1 had a similar effect as shRNA double knockdown of NEIL1/CSB. This may indicate complete lack of repair of induced Fapys in the absence of sufficient NEIL1.

**DISCUSSION**

It has been proposed that the symptoms of CS may reflect high levels of oxidative damage in neural and other tissues and that CSB may play a role in the repair of oxidative DNA damage. These ideas are consistent with the observations that CSB-deficient cells are hypersensitive to oxidative stress (52), accumulate oxidatively induced DNA lesions, and are genetically unstable (for review, see Ref. 53). However, the role of CSB in molecular mechanisms underlying CS phenotypes remains unclear. A direct role for CSB in lesion bypass by RNA polymerase II (54) may underlie some of the neurodegenerative aspects...
of the disease, but it does not explain the accumulation of oxidatively induced DNA lesions and mutations. Here we show that endogenous FapyGua and FapyAde accumulate in DNA from brain and kidney and that FapyAde accumulates in liver nuclear and mtDNA of csb<sup>−/−</sup> mice (Fig. 1). The role of CSB in the repair of these lesions was confirmed by the observation that shRNA knockdown of NEIL1/Csb completely inhibited repair of menadione-induced FapyGua in HeLa cells (Fig. 7B). As reported previously (21), the level of 8-OH-Gua is also higher in brain and kidney DNA of csb<sup>−/−</sup> mice than in wt mice (Fig. 1A) (20). These results indicate that functional CSB protein is directly involved in the repair of these lesions and suggest that elevated levels of FapyGua and FapyAde may play a role in the pathophysiology of CS. The significant accumulation of these lesions in the brains of the csb<sup>−/−</sup> mice more specifically may indicate a causative role in the neurodegeneration observed in CS patients.

FapyGua and FapyAde are substrates for mouse and human NEIL1 (27–29). Importantly, the results presented here demonstrate a functional and physical interaction between CSB and NEIL1 in vivo and in vitro (Figs. 2, 3, and 6). CSB stimulation of NEIL1 incision was greater at higher CSB:NEIL1 ratios (Fig. 2B), and immunostaining of HeLa cells show that CSB and NEIL1 co-localize in the nucleoplasm (Fig. 5C). NEIL1 was also detected in the cytosol, suggesting possible localization to mitochondria (30). Although CSB did not co-localize with NEIL1 in the cytosol under the conditions used here, the observation that FapyAde accumulates in liver mtDNA in csb<sup>−/−</sup> mice may suggest that a NEIL1/CSB interaction may take place in mitochondria as well. Previous studies suggest that CSB redistributes in cells exposed to H<sub>2</sub>O<sub>2</sub> and that this redistribution requires c-Abl-induced phosphorylation of CSB (55). Thus, CSB may be recruited to oxidatively induced DNA lesions, where the NEIL1/CSB interaction is functionally relevant. The role of NEIL1/CSB may be more critical for repairing FapyAde than FapyGua and 8-OH-Gua because OGG1 does not repair FapyAde (44) and because NEIL1 is the only enzyme known to repair FapyAde in human cells.

Interestingly, CSB may stimulate NEIL1 incision in a lesion-specific manner because the stimulation occurs in reactions with FapyGua, FapyAde, and 5-OH-Ura but not in reactions with 8-OH-Gua. However, 8-OH-Gua is a relatively poor substrate for NEIL1, especially when the DNA substrate contains multiple lesions. CSB does not stimulate NEIL1 incision in a partial ssDNA bubble structure (Fig. 3B), and CSB does not stimulate incision of Fapys by E. coli Fpg. These results underscore the specificity in the interaction between CSB and NEIL1.

By quantifying the covalent intermediate during NEIL1-catalyzed DNA strand cleavage, we demonstrated that CSB stimulates strand cleavage by NEIL1 (Fig. 4). We also showed that base release by NEIL1 is not uncoupled from strand cleavage either in the presence or absence of CSB (supplemental Fig. S3). Although these reactions were not performed under single turnover conditions (25 fmol of enzyme and 50 fmol of DNA substrate), the results suggest that CSB alters the kinetic properties of NEIL1.

To map the functional interaction between CSB and NEIL1 more specifically, we used CSB protein fragments and CSB point mutant proteins (Fig. 5 and supplemental Fig. S4). Our result show that the N-terminal CSB (2–341) fragment stimulates NEIL1 incision activity to the same degree as full length protein and that no stimulation is seen using the CSB (514–986) fragment, containing the ATPase domain. CSB ATPase mutant (CSB E646Q) and domain VI mutant (CSB Q942E) proteins stimulated the incision activity of NEIL1 similarly to the CSBwt, indicating that the ATPase activity of CSB is not required for its function on NEIL1 activity. A connection between the N-terminal domain of CSB and BER is also suggested by results showing that CSB interacts with PARP1 through the CSB (2–341) fragment and that the main site for poly(ADP-ribosyl)ation is situated within the CSB (2–341) fragment (24). Hence, our result further strengthens the connection between the N-terminal part of CSB, but not the ATPase domain, and CSB function in BER. Furthermore, these are the first results showing a direct stimulation of a BER protein by a specific fragment of CSB.

The involvement of CSB in both NER and BER could be speculated to be mediated by one common or two separate mechanisms. We reported earlier that the ATPase activity is needed for the role of CSB in repair of UV lesions and transcription (40, 41) but not for its role in BER (18, 19). Together with the results presented here showing that the ATPase domain is also dispensable for CSB stimulation of NEIL1 activity, these observations suggest that CSB involvement in the two repair pathways is independent or at least is not mediated by the exact same mechanism. The poly(ADP-ribosyl)ation of CSB is increased after oxidative stress, and the fact that this modification results in a
decrease of ATPase activity further suggests that the ATPase activity of CSB is not involved in the BER (24).

In summary, this study demonstrates a functional interaction between CSB and NEIL1 and that the CSB is in complex with NEIL1 in situ. This interaction is likely to play an important role in repair of endogenous and induced Fapy in vivo. Recent studies show that mice lacking NEIL1 have a complex severe phenotype resembling the metabolic syndrome in humans. The results presented here are consistent with the hypothesis that higher than normal levels of FapyAde and FapyGua may have severe biological consequences and that unrepaired or misrepaired FapyAde and FapyGua may contribute to the pathology of CS.

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