Mutant Cockayne syndrome group B protein inhibits repair of DNA topoisomerase I-DNA covalent complex

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Two UV-sensitive syndrome patients who have mild photosensitivity without detectable somatic abnormalities lack detectable Cockayne syndrome group B (CSB) protein because of a homozygous null mutation in the \textit{CSB} gene. In contrast, mutant CSB proteins are produced in CS-B patients with the severe somatic abnormalities of Cockayne syndrome and photosensitivity. It is known that the piggyBac transposable element derived 3 is integrated within the \textit{CSB} intron 5, and that \textit{CSB}-piggyBac transposable element derived 3 fusion (CPFP) mRNA is produced by alternative splicing. We found that CPFP or truncated CSB protein derived from CPFP mRNA was stably produced in CS-B patients, and that wild-type CSB, CPFP, and truncated CSB protein interacted with DNA topoisomerase I. We also found that CPFP inhibited repair of a camptothecin-induced topoisomerase I-DNA covalent complex. The inhibition was suppressed by the presence of wild-type CSB, consistent with the autosomal recessive inheritance of Cockayne syndrome. These results suggested that reduced repair of a DNA topoisomerase I-DNA covalent complex because of truncated CSB proteins is involved in the pathogenesis of CS-B.

Introduction

Nucleotide excision repair (NER) is a versatile DNA repair system that removes a wide range of DNA lesions including UV damage (Friedberg et al. 2006). There are two subpathways in NER. One is transcription-coupled NER (TC-NER), which preferentially removes transcription-blocking DNA damage from the transcribed strand in active genes. Another is global genome NER (GG-NER), which removes lesions throughout the genome (Hanawalt & Spivak 2008). There are two autosomal recessive disorders that are specifically deficient in TC-NER: Cockayne syndrome (CS) and UV-sensitive syndrome (UVS). CS is characterized by photosensitivity and abnormalities in physical and neurologic development such as postnatal growth failure, cachectic dwarfism, mental retardation, pigmented retinopathy, deafness, birdlike face, and short life span (approximately 10 years). However, patients with CS have no predisposition to
skin cancer (Nance & Berry 1992). CS is classified into two genetic complementation groups (CS-A and CS-B). CS-A and CS-B cells exhibit a defect in recovery of RNA synthesis after UV irradiation (UV-RRS) and a defect of TC-NER of UV damage, but have proficient GG-NER (Mayne & Lehmann 1982; Venema et al. 1990). On the other hand, UV’S patients show photosensitivitiy and mild freckling with no skin tumors. However, in contrast to patients with CS, UV’S patients have no neurologic or somatic abnormalities. Cells from UV’S patients exhibit UV hypersensitivitiy and are deficient in UV-RRS and TC-NER of UV damage but are proficient in GG-NER, as are CS-B cells (Fujiwara et al. 1981; Itoh et al. 1995; Spivak et al. 2002).

The Cockayne syndrome group B (CSB) gene encodes a protein of 1493 amino acids. We have reported that one UV’S patient, UV’S1KO, has a homozygous nonsense mutation at amino acid position 77 (77Arg to stop) in the CSB gene and that no CSB proteins were detected in UV’S1KO cells. In contrast, stable truncated CSB proteins are produced in cells from CS-B patients and are localized in the cell nucleus (Horibata et al. 2004). Based on these findings, we hypothesized that the mutant CSB proteins have an inhibitory function in the nucleus that leads to CS-features, whereas there may be no such inhibition and consequently no somatic CS-features in UV’S1KO.

In order to corroborate our hypothesis, we first examined the structure of truncated CSB proteins produced in CS-B cells. It is known that the piggyBac transposable element derived 3 (PGBD3) is inserted in human CSB intron 5, and PGBD3 sequences are recognized as an exon owing to alternative splicing (Newman et al. 2008). In this study, we found that the fusion mRNA including CSB exons 1–5 and the PGBD3 exon encodes a CSB-PGBD3 fusion protein (CPFP) in normal human and several CS-B patients, and a truncated CSB protein 3PV-CSB in particular CS-B cells (CS3PV). We also found that wild-type CSB protein (WT-CSB), CPFP, and 3PV-CSB interacted with DNA topoisomerase I (Top1). Importantly, CPFP inhibited the repair of camptothecin (CPT)-induced Top1-DNA covalent complexes, and the inhibition was suppressed by the concomitant presence of WT-CSB. These results suggest that the inhibition of repair of Top1-associated DNA lesions by truncated forms of CSB is at least one of the causes of somatic CS-features in CS-B patients that are not observed in UV’S patient.

Results
Stable production of truncated CSB proteins in CS-B cells

The CSB gene mutations in UV’S and CS-B patients’ cells are shown in Fig. 1A and listed in Table S1 (Supporting Information). Western blot analysis of cell lysates of primary fibroblast cells from UV’S and CS-B patients with an anti-CSB antibody showed that in normal human cells (FS3), wild-type CSB protein (WT-CSB), and truncated CSB proteins with molecular weights of about 150 and 30 kDa, which are designated as p150 and p30, respectively, were detected. In CS-B cells, WT-CSB was absent, whereas p150 and p30 were detected (Fig. 1B). In CS3PV cells (Colella et al. 1999), the p150 was missing, but truncated CSB protein with an apparent MW of ≈70 kDa was detected (Fig. 1B). On the other hand, no CSB proteins were detected in UV’S1KO and CS3AM cells. CS3AM, a Japanese patient who shows only mild photosensitivity and freckles but no neurologic abnormality, was originally identified as an atypical CS-B patient (Miyachi-Hashimoto et al. 1998) and has the same homozygous nonsense mutation at amino acid position 77 (77Arg to stop) in the CSB gene as UV’S1KO (Fig. 1B, Fig. S1 and Data S1 in Supporting Information).

In silico analysis of the CSB gene locus showed that a piggyBac transposable element derived 3 (PGBD3) sequence is located in CSB intron 5 (Fig. 1A). According to the NCBI database, PGBD3 is recognized as a novel exon (NCBI ID BC034479) by alternative splicing, resulting in the production of N-terminal CSB/PGBD3 fusion protein (CPFP: N-terminal CSB1−763 and piggyBac) (Fig. 1C). To confirm that p150 was derived from CPFP mRNA, we performed RNA interference analysis with siRNA targeted for various CSB and PGBD3 sequences (Fig. 1C,D). Transfection of siRNA for the 5’-region of CSB mRNA (siCSB/CPFP) resulted in the decrease of both WT-CSB and p150 in HeLa cells. Transfection of siRNAs for the 3’-regions of CSB mRNA (siCSB−1, −2 and −3) decreased only WT-CSB, whereas siRNAs for PGBD3 (siPGBD3−1 and −2) decreased only p150. Transfections with all the siRNAs for CSB and PGBD3 resulted in the decrease of both WT-CSB and p150. Taken together, these results indicated that p150 corresponds to CPFP.

The expression of WT-CSB, p150, and truncated CSB protein was examined in FS3, CS1AN, and CS3PV cells transfected with siPGBD3−1 and 3–2, respectively. As shown in Fig. 2A, p150 was

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significantly decreased in FS3 and CS1AN cells transfected with siPGBD3-1. In addition, the truncated CSB protein with an apparent MW of 70 kDa was also decreased in CS3PV cells when transfected with siPGBD3-1. These results indicated that p150 was derived from CPFP mRNA in WT and CS-B cells, and that the 70 kDa truncated CSB protein in CS3PV cells (3PV-CSB) was also derived from CPFP mRNA with a nonsense mutation at amino acid position 453 (453Arg to stop) (Fig. 2B). Consistent with these results, Western blot analysis of CPFP with anti-PGBD3 antibody in normal and CS-B cells indicated that p150 contains PGBD3 protein in these cells (Fig. S2 in Supporting Information).

**Purification of WT-CSB, CPFP, and 3PV-CSB protein complexes**

In order to test for an inhibitory function of CPFP and 3PV-CSB, we examined protein complex formation with WT-CSB, CPFP, and 3PV-CSB. HEK293 cell lines that stably express the C-terminally FLAG-6xHis-tagged WT-CSB, CPFP, and 3PV-CSB, respectively were established. Whole-cell extracts of these cell lines were subjected to affinity purifications using a nickel chelate and anti-FLAG antibody, and the purified WT-CSB, CPFP, and 3PV-CSB protein complexes were analyzed by SDS–PAGE and visualized with silver staining (Fig. 3A). Mass spectrometric
and Western blot analyses of each purified complex showed that all of the CSB protein complexes contained DNA topoisomerase I (Top1) (Fig. 3B,C). To confirm the interaction between CSB and Top1, we carried out the reciprocal experiment by establishing a HEK293 cell line stably expressing the HA-FLAG-tagged Top1 (HAF-Top1). Whole-cell extracts from these cells were immunoprecipitated by anti-FLAG antibody, and the elutants were subjected to SDS–PAGE, followed by Western blotting using the anti-CSB antibody. As shown in Fig. 3D, WT-CSB and CPFP were bound to Top1.

We then examined whether Top1 directly interacts with WT-CSB, CPFP, and 3PV-CSB proteins. The purified WT-CSB and HAF-Top1 proteins were active in DNA-dependent ATPase and topoisomerase assays, respectively (Fig. S3 and Data S1 in Supporting Information). WT-CSB, CPFP, and 3PV-CSB proteins were immobilized onto nickel Sepharose beads and incubated with HAF-Top1. After washing with buffer, the bound proteins were analyzed by SDS–PAGE and Western blotting using anti-HA antibody. As shown in Fig. 3E, WT-CSB, CPFP, and 3PV-CSB proteins directly bound Top1. Taken together, these results indicate that WT-CSB, CPFP, and 3PV-CSB can each interact with Top1 in human cells.

**Subcellular distribution of Top1, WT-CSB, and CPFP**

To verify the interaction between Top1 and WT-CSB or CPFP in vivo, we examined the subcellular distribution of these proteins using confocal fluorescent microscopy. CS3AM primary cells were co-transfected with the N-terminally EGFP-tagged Top1 (EGFP-Top1) and the C-terminally DsRed-Monomer-tagged WT-CSB (WT-CSB-DsRed) or CPFP (CPFP-DsRed). The predominant accumulation of both EGFP-Top1 and WT-CSB-DsRed was detected in the nucleoli, in addition to a homogeneous distribution in the nucleoplasm (Fig. 3F). In contrast, the major population of CPFP-DsRed distributed in the nucleoplasm (Fig. 3G). These in vivo data suggest that WT-CSB associates with Top1 both in nucleoli and nucleoplasm, whereas CPFP associates with Top1 mainly in nucleoplasm.

It is known that Top1 rapidly moves from nucleoli to nucleoplasm after treatment of the cells with anti-cancer drug camptothecin (CPT) (Leppard & Champoux 2005). In order to determine whether WT-CSB and CPFP show dynamic distribution as well as Top1 after CPT treatment, we examined the subcellular distribution of EGFP-Top1, WT-CSB-DsRed, and CPFP-DsRed in the CPT-treated cells. Both
Figure 3  WT-CSB, CPFP, and 3PV-CSB form protein complexes containing DNA topoisomerase I. (A) Silver staining of the protein complexes purified from whole-cell extracts of HEK293 cells expressing His-FLAG-tagged WT-CSB, CPFP, and 3PV-CSB proteins, respectively, by affinity purification using nickel agarose beads and anti-FLAG antibody. As a control, a mock purification was performed with whole-cell extracts from non-transfected HEK293 cells. Mass spectrometric analyses showed that all the Cockayne syndrome group B (CSB) protein complexes contain Top1 (indicated by arrowheads). (B) Protein complex purified from HEK293 cells expressing WT-CSB, CPFP, and 3PV-CSB were analyzed by Western blotting with antibody against CSB, respectively. (C) The whole-cell extracts of HEK293 cells expressing WT-CSB, CPFP, and 3PV-CSB were analyzed by Western blotting with antibody against Top1. Note that Top1 was detected in all cell extracts (lane 1, 3, 5 and 7) and in the WT-CSB, CPFP, and 3PV-CSB protein complexes (lane 4, 6 and 8) but not in a mock purification (lane 2). (D) Whole-cell extracts of HEK293 cells expressing HA-FLAG-tagged Top1 or vector alone were immunoprecipitated by anti-FLAG antibody, subjected to SDS–PAGE and Western blotting with anti-CSB antibody. Note that both CSB and CPFP were detected in the whole-cell extracts (lane 1 and 2) and in the Top1-immunoprecipitates (lane 4) but not in mock-immunoprecipitates (lane 3). (E) Direct binding of CSB and Top1. WT-CSB-HF (C-terminally His-FLAG-tagged WT-CSB), CPFP-HF and 3PV-CSB-HF adsorbed to Ni-Sepharose were incubated with purified HA-FLAG-Top1. The bound proteins were analyzed by Western blotting with anti-HA antibody. (F, G) Subnuclear localization of EGFP-Top1, CSB-DsRed, and CPFP-DsRed in CS3AM primary fibroblasts. Note that both EGFP-Top1 and WT-CSB-DsRed predominantly accumulated in nucleoli and distributed homogeneously in nucleoplasm, whereas only few CPFP-DsRed accumulated in nucleoli, and majority of CPFP-DsRed distributed in nucleoplasm. (H, I) Subnuclear localization of Top1, WT-CSB, and CPFP in the camptothecin (CPT)-treated CS3AM primary fibroblasts. CS3AM cells transfected with EGFP-Top1 and CSB-DsRed or CPFP-DsRed were treated with CPT (25 μM, 1 h) and fixed. Note that EGFP-Top1, CSB-DsRed, and CPFP-DsRed in nucleoli relocated in nucleoplasm after CPT treatment.

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EGFP-Top1 and WT-CSB-DsRed rapidly moved from nucleoli to nucleoplasm after CPT treatment (Fig. 3H). CPFP-DsRed remained in nucleoplasm after CPT treatment (Fig. 3I). These in vivo data indicate that CSB and CPFP interact with Top1 in the nucleoplasm of the CPT-treated cells.

Repair of Top1–DNA covalent complex in the CPFP-expressing CS-B cells

Top1 reversibly nicks DNA and relaxes superhelical tension in DNA induced during replication and transcription. In the process, it generates a reversible and transient intermediate known as Top1–DNA covalent complex (Top1-cc), in which Top1 is covalently attached via a tyrosyl residue (human Tyr723) to the 3′-terminus of a single-stranded nick. Following the release of torsional stress, Top1 resales the nick and restores the integrity of the double helix by re-ligation (Champoux 2001). CPT is known to block the re-ligation reaction of Top1, resulting in accumulation of Top1-cc.

These considerations prompted us to perform an immunocomplex of enzyme (ICE) assay (Muller et al. 1985; Miao et al. 2006) to examine whether the repair of CPT-induced Top1-cc is decreased in hTERT-immortalized CS-B fibroblast cells expressing CPFP, when compared with the isogenic CS-B cells in which CPFP was downregulated. Because it has been reported that the repair of CPT-induced Top1-cc was defective in SV40-transformed cells and some kinds of cancer cells (Desai et al. 2001, 2003), we used primary fibroblast cells and hTERT-immortalized cells derived from normal human and CS-B patients for the measurement of repair of Top1-cc. For ICE assay, cells were treated with CPT, and then lysed. DNA–protein complex was isolated from cell lysates by centrifugation (Champoux 2001). CPT is known to block the re-ligation reaction of Top1, resulting in accumulation of Top1-cc.

TelCOFS02MA is an hTERT-immortalized CS-B fibroblast cell line (Meira et al. 2000) derived from a patient with cerebro-oculo-facio-skeletal syndrome (COFSS) and has a homozygous mutation in CSB gene: 3794–3795-bp deletion leading to a frame shift followed by stop codon at the 1240 amino acid residue (Fig. 1A and Table S1 (Supporting Information)). The phenotype of COFSS is very similar to CS, including abnormal facies, microcephaly, and a similar pattern of neurologic abnormalities (Friedberg et al. 2006). TelCOFS02MA cells produce only CPFP and p30 (Fig. 1B). To confirm the inhibitory function of CPFP in the repair of CPT-induced Top1-cc, the amount of Top1-cc was measured in TelCOFS02MA cells in which CPFP was knocked down by siPGPD3-2 (Figs 4A, 1C). TelCOFS02MA cells transfected with siLucGL3 were established as a control (Fig. 4A). As shown in Fig. 4B,C, the amount of Top1-cc induced by the treatment with 2 μM CPT for 1 h was decreased in the CPFP-knockdown TelCOFS02MA cells, when compared with the siLucGL3 transfected control cells. Then, we examined whether the inhibition of repair of CPT-induced Top1-cc because of CPFP is suppressed by the expression of exogenous WT-CSB in TelCOFS02MA cells (Fig. 4D). The amount of CPT-induced Top1-cc was decreased in TelCOFS02MA cells expressing WT-CSB cells, compared with those expressing vector alone (Fig. 4E,F). Taken together, these results indicate that CPFP inhibited the repair of CPT-induced Top1-cc, but the inhibition was suppressed by the concomitant presence of WT-CSB.

The increased amount of CPT-induced Top1-cc in CPFP-expressing CS-B cells could be caused by not only a defect in repair of Top1-cc, but also increased formation of Top1-cc because WT-CSB and CPFP are bound to Top1. In order to examine which explanation is more plausible for the increased Top1-cc in CS-B cells, we measured Top1-cc in CPFP-knockdown and control TelCOFS02MA cells that were incubated for 5 h after CPT treatment. Cells were treated with 2 μM CPT for 6 h. In the 6 h CPT treatment, the amount of Top1-cc was similar in CPFP-knockdown cells and in control cells at 0-h incubation (Fig. 4G,H), indicating saturation of Top1-cc induced by long exposure of CPT. However, the amount of Top1-cc was much lower in CPFP-knockdown TelCOFS02MA cells than in control cells after 5-h incubation (Fig. 4G,H). These results indicate that the increased amount of Top1-cc in CPFP-expressing CS-B cells is mainly because of a defect in repair of CPT-induced Top1-cc.

CPT-induced inhibition of transcription in CPFP-knockdown and control TelCOFS02MA cells

It is known that when RNA polymerase II (RNAP2) is encountered with Top1-cc, transcription is rapidly arrested, and then restored on repair of Top1-cc (el-Khamisy & Caldecott 2007). Therefore, we measured a RNA synthesis after CPT treatment in the CPFP knockdown and control TelCOFS02MA cells. As shown in Fig. 5A, RNA synthesis was markedly decreased in both CPFP knockdown and control cells immediately after CPT treatment (0 h), and further
Figure 4 Camptothecin (CPT)-induced Top1-cc was decreased by the knockdown of CPFP (p150) or introduction of WT-CSB in CS-B cells. (A) Knockdown of CPFP (p150) by transfection with siPGBD3-2 in TelCOFS02MA (CS-B) cells. siLucGL3 was used as a negative control. (B) ICE assay in CPFP-knockdown and control TelCOFS02MA (CS-B) cells. Forty-eight hours after the secondary transfection of siRNA, cells were treated with 2 μM CPT for 1 h, and ICE assay using different amounts of DNA (300–1200 ng) was performed to measure Top1-cc. The Top1-cc was measured by Western blotting of DNA-containing fractions with anti-Top1 antibody. (C) Quantitative measurement of Top1-cc in (B). Amount of Top1-cc was expressed after standardization of the amount of Top1-cc in 1200 ng DNA fraction derived from siLuc as 100%. Error bars represent the SD from the mean of three independent experiments. (D) Expression of WT-CSB in TelCOFS02MA (CS-B) cells transfected twice with HA-FLAG-tagged WT-CSB cDNA or vector alone. (E) ICE assay in TelCOFS02MA (CS-B) cells expressing WT-CSB shown in (D). Twenty-four hours after the secondary transfection of WT-CSB cDNA or vector alone into TelCOFS02MA (CS-B) cells, cells were treated with 2 μM CPT for 1 h, and ICE assay using different amounts of DNA (75–1200 ng) was performed. (F) Quantitative measurement of Top1-cc in (E). Amount of Top1-cc was expressed after standardization of the amount of Top1-cc in 1200 ng DNA fraction derived from cells transfected with vector alone as 100%. Error bars represent the SD from the mean of three independent experiments. (G) CPFP-knockdown and control TelCOFS02MA (CS-B) cells were treated with 2 μM CPT for 6 h, and then incubated for 0 h (no repair) or 5 h after CPT treatment. ICE assay using different amounts of DNA (78–2500 ng) was performed and the Top1-cc was measured by Western blotting of DNA-containing fractions with anti-Top1 antibody. Note that the amount of Top1-cc was saturated at 0 h after treatment with 2 μM CPT for 6 h, but the difference in the amount of Top1-cc between CPFP-knockdown and control CS-B cells became evident during incubation for 5 h after CPT treatment, indicating that CPFP inhibited a repair of CPT-induced Top1-cc. (H) Quantitative measurement of Top1-cc in (G). Amount of Top1-cc was expressed after standardization of the amount of Top1-cc in 2500 ng DNA fraction derived from siLuc – no repair as 100%.
decreased 2 h after CPT treatment. Then, the RNA synthesis recovered in CPFP-knockdown and control TelCOFS02MA cells, but the recovery was greater in CPFP-knockdown cells compared with control cells (Fig. 5A). These results indicate that the recovery of RNA synthesis after CPT treatment is inhibited by the presence of CPFP.

**CPFP inhibits the repair of CPT-induced single-strand DNA breaks**

Camptothecin-induced Top1-cc generates Top1-concealed single-strand DNA breaks (Top1-SSBs) by collision with RNAP2 or some types of DNA lesions. In order to validate that CS-B cells expressing CPFP have a defect in repair of CPT-induced Top1-cc, we measured the CPT-induced Top1-SSBs in the CPFP-knockdown and control TelCOFS02MA cells. CPFP-knockdown and control TelCOFS02MA cells were treated with CPT (2.5 μM), and then SSBs were directly measured by the alkaline comet assay. As shown in Fig. 5B, the mean comet tail moment was significantly increased in the presence of MG-132.
cells transfected with control siRNA, whereas the increase was significantly less in CPFP-knockdown cells, both immediately and 1 h after CPT treatment. These results indicate that repair of CPT-induced Top1-SSBs is inhibited by the presence of CPFP.

CPT-induced degradation of Top1 in CPFP-knockdown and control TelCOFS02MA cells

It was reported that the repair of Top1-cc after CPT treatment is correlated with degradation of Top1 and the largest subunit (Rpb1) of RNAP2 by 26S proteasome (Desai et al. 2003), and that Rpb1 is hyperphosphorylated in HCT116 cells treated with CPT (Sordet et al. 2008). In order to address the possible function of CPFP protein in the repair of Top1-cc, the CPT-induced hyperphosphorylation of Rpb1, and degradation of Rpb1 and control TelCOFS02MA cells. In our experimental conditions using TelCOFS02MA cells, the major population of Rpb1 was hyperphosphorylated before CPT treatment, therefore no significant shift from hypophosphorylated form (RNAP2a) to hyperphosphorylated form (RNAP2o) after CPT treatment could be observed in either CPFP-knockdown or control TelCOFS02MA cells. In addition, degradation of RNAP2o was not observed (Fig. 5C). On the other hand, we observed CPT-induced degradation of Top1 that was inhibited by the presence of MG-132, a proteasomal inhibitor. However, no difference in the degradation profile of Top1 was detected in CPFP-knockdown and control TelCOFS02MA cells in the absence and presence of MG-132 (Fig. 5C,D). These results suggest that the presence of CPFP does not affect the degradation of Top1 by proteasome following CPT treatment.

Discussion

Both UVs and CS can result from mutations in the CSB gene. Here, we show that truncated CSB proteins are produced in cells from different CS-B patients, whereas no CSB protein is produced in two UVs patients, and that these truncated proteins interact with Top1 and inhibit the repair of CPT-induced Top1-associated lesions. Importantly, WT-CSB prevented the effects of the truncated CSB proteins on Top1-associated lesions, consistent with the known recessive inheritance of CS. These observations indicate that the decreased repair of Top1-associated lesions can cause at least a subset of the somatic symptoms observed in CS-B patients.

Stable production of truncated CSB proteins due to insertion of piggyBac transposon

It is known that most mRNAs with a premature termination codon are targeted for nonsense-mediated mRNA decay (NMD), whereas mRNAs containing a premature termination codon located <50–55 nucleotides upstream of the ‘3’-most exon–exon junction or downstream of this junction escapes NMD (Lejeune & Maquat 2005). Accordingly, a CPFP mRNA with a nonsense mutation within 50–55 nucleotides upstream of the junction of CSB exon 5 and PGBD3 should not be targeted to NMD, leading to the production of the N-terminal portion of CSB protein using the non-canonical stop codon in CSB exon 5. CS3PV cells, which are derived from a CS-B patient with CS-features, have a homozygous C1436T nonsense mutation (Arg to stop) in CSB exon 5 that is located 40 nucleotides upstream of the junction of exon 5 and PGBD3 (Fig. 2B). Therefore, CPFP mRNA containing this nonsense mutation in exon 5 is not targeted for NMD in CS3PV cells, whereas the full-length spliced CSB mRNA encoding all 21 exons and the same nonsense mutation (C1436T transition) is targeted for NMD (Fig. 2B). As a consequence, the truncated CSB protein (3PV-CSB; N-terminal CSB1) is stably produced in CS3PV cells (Fig. 2B). Thus, the insertion of piggyBac sequences into the CSB gene plays a critical role in the production of stable N-terminal truncated CSB proteins in CS-B cells, even when the PGBD3 coding sequences are not translated into protein, as in CS3PV cells. Furthermore, the presence of typical CS features in patient CS3PV, despite the fact that the PGBD3 sequences are not translated into protein in the patient’s cells (Fig. 2A,B), suggests that the PGBD3 protein per se does not play a role in the CS pathogenesis.

Retarded repair of Top1–cc in CPFP-expressing CS-B cells

Mass spectrometry and Western blot analysis showed that each complex of WT-CSB, CPFP, and 3PV-CSB proteins contained Top1, and they directly interacted (Fig. 3E). In addition, confocal fluorescent microscopy showed that Top1, WT-CSB, and CPFP located in the nucleoplasm after CPT treatment (Fig. 3H,I). The CSB–Top1 interaction prompted us to examine whether the mutant CSB proteins consisting of the N-terminal portions of CSB may inhibit repair of CPT-induced Top1-cc. Using the ICE assay,
we found that knockdown of CPFP in TelCOFS02MA (CS-B) cells resulted in the decreased amount of Top1-cc (Fig. 4A–C), and the amount of CPT-induced Top1-cc was decreased in TelCOFS02MA cells by the expression of exogenous WT-CSB (Fig. 4D–F). We also found that CPT-induced Top1-cc increased in primary CS3AM cells expressing CPFP or 3PV-CSB when compared with those in CS3AM cells expressing WT-CSB or vector alone (Fig. S4 in Supporting Information).

Moreover, the recovery of RNA synthesis after CPT treatment (Fig. 5A) and the repair of Top1-concealed single-strand DNA breaks (Fig. 5B) was increased in the CPFP-knockdown CS-B cells.

Taken together, these results indicate that CPFP inhibited the repair of CPT-induced Top1-cc, but the inhibition was suppressed by the concomitant presence of WT-CSB, consistent with recessive inheritance of CS-B.

Possible role of CSB proteins in Top1-cc repair and implication for CS-B

Repair of Top1-cc is divided in the repair/excision (3′-end processing) of the Top1 covalently linked to the DNA and repair/religation of the 5′ end. The most well-known enzyme for 3′-end processing is tyrosyl-DNA-phosphodiesterase (Tdp1). In addition, it has been known that alternative pathway involving 3′-flap endonuclease such as Mus81/Eme1, Mre11/Rad50, and XPF/ERCC1 are also involved in these pathways (Pommier 2009). We propose in the manuscript that there is also a CSB-dependent pathway to repair the Top1-cc.

It was reported that the repair of Top1-cc after CPT treatment is correlated with degradation of Top1 and Rpb1 of RNAP2 by 26S proteasome (Desai et al. 2003), and restoration of CPT-induced inhibition of RNA synthesis (el-Khamisy & Caldecott 2007). In our experimental conditions using TelCOFS02MA cells, degradation of RNAP2o was not observed (Fig. 5C), whereas CPT-induced degradation of Top1 that was inhibited by the presence of MG-132, a proteasomal inhibitor, was detected (Fig. 5D). However, no difference in the degradation profile of Top1 was detected in CPFP-knockdown and control TelCOFS02MA cells in the absence and presence of MG-132 (Fig. 5C,D). These results suggested that CPFP does not affect the degradation of Top1 by proteasome following CPT treatment. On the other hand, the recovery of RNA synthesis after CPT treatment and repair of Top1-concealed single-strand breaks was greater in CPFP-knockdown cells compared with control cells (Fig. 5A,B). Taking into account these results, we hypothesize that the residual C-terminal Top1 polypeptide containing the Tyr723 residue remains covalently linked to the 3′-phosphoryl termini of single-strand nicks via a tyrosyl phosphate bond and results in the inhibition of resumption of RNA synthesis after CPT treatment in CS-B cells. Consistent with this hypothesis, the C-terminal region of Top1 (peptide F and G in Fig. S5A–C in Supporting Information) is bound to WT-CSB, CPFP, and 3PV-CSB proteins, respectively (Fig. S5E in Supporting Information). In addition, anti-Top1 antibody used in our study recognized the C-terminal portion of Top1 (Fig. S5D in Supporting Information).

Top1-cc can result from many different endogenous DNA lesions including abasic sites, strand breaks, and oxidative DNA lesions (Pourquier & Pommier 2001). Therefore, it is likely that there is a steady-state level of Top1-cc in cells that results in transcription defects and is subject to repair. If so, a transcription abnormality in response to endogenous Top1-cc, resulting from truncated CSB proteins in CS-B cells as we have shown here, could explain some of the CS features in CS-B patients. The neurologic abnormalities in CS include microcephaly, myelination defects, and cerebellar degeneration (Weidenheim et al. 2009). Specifically, the cerebellar Purkinje neurons undergo severe degeneration and atrophy in patients with CS (Brooks et al. 2008). Purkinje neurons in the human cerebellum have very high levels of Top-1 compared to other neurons in the brain (Gorodetsky et al. 2007). High levels of Top1 in Purkinje neurons would put these cells at greater risk for Top1-cc, which could explain why these cells are more severely affected by the production of truncated CSB proteins in CS-B patients than cells with less Top1.

Cockayne syndrome patients with mutations in the CSA gene have essentially the same phenotype as those with mutations in CSB. Both CS-A and CS-B cells are hypersensitive to CPT and also defective in the recovery of RNA synthesis after CPT treatment (Squires et al. 1993; Desai et al. 2003). However, in our present experimental condition, CS-A cells show normal repair of Top1-cc after CPT treatment (Fig. S6 in Supporting Information). The mechanistic basis for this feature in CS-A cells remains to be clarified.

Two patients (CS548VI and CS539VI) from Reunion Islands, who showed somatic and neuro-
logic CS-features and died at the age of 6 and 8, respectively, had a homozygous deletion in the exon1 and upstream regulatory region of the CSB gene (Laugel et al. 2008). It was reported that no WT-CSB, CPFP, or smaller CSB polypeptides were detected in these CS-B cells. Although it remains to analyze whether these CS-B patients had an abnormality in the repair of Top1-cc, given the potential for founder mutations in the native Reunion Island population (Laugel et al. 2008), it is possible that they have been homozygous for a mutation in a separate gene that, in combination with the absence of CSB, results in early onset CS features.

It has been reported that CS-B cells are hypersensitive to treatment with H$_2$O$_2$, whereas UV'S (Kps3) cells exhibited normal resistance. Host cell reactivation assay with lacZ expression plasmids containing the oxidative base lesions showed that CS-B cells are defective in recovery of expression, whereas UV'S cells show normal level of expression. In addition, repair of oxidative DNA damage is deficient in CS-A and CS-B cells (Tuo et al. 2003; de Waard et al. 2004; Thorslund et al. 2005; Spivak & Hanawalt 2006; Trapp et al. 2007). It was recently shown that a UV'S patient (UV1VI) has a mutation in the CSA gene and showed normal sensitivity to oxidative DNA damage, whereas CS-A patient's cells are hypersensitive to oxidative stress (Nardo et al. 2009). Although the exact role of CSA and CSB in oxidative damage repair is not known, defective repair of Top1-cc and of oxidative DNA damage in CS cells are not mutually exclusive; in fact, oxidative DNA lesions can cause Top1-cc.

**Experimental procedures**

**Cell strains**

Cell strains used were described previously (Fujiwara et al. 1981; Miyauichi-Hashimoto et al. 1998; Colella et al. 1999; Meira et al. 2000). A primary fibroblast cell strain derived from normal human skin, FS3 was established in our laboratory. Primary UV'S fibroblast cells, UV'S1KO, and CS3AM were kindly provided by Yoshisada Fujiwara and Takeshi Horio, respectively. Primary and hTERT immortalized CS-B fibroblast cell strains, TelCOFS03MA and TelCOFS02MA, were kindly provided by Errol C. Friedberg. The primary CS-B fibroblast cell strain CS3PV was kindly provided by Miria Stefanini. Primary CS-B fibroblast cell strains, CS1AN (GM00739), CS1BE (GM01629), and CS7SE (GM01428) were obtained from the NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden N.J. The primary CS-A fibroblast cell strain Mps1 was described elsewhere (Ren et al. 2003).

**SDS–PAGE and immunoblotting**

SDS–PAGE and immunoblotting were performed as described previously (Horibata et al. 2004). Whole-cell extracts were separated by SDS–PAGE and transferred to PVDF membranes in blotting buffer (25 mm Tris–HCl pH 8.3, 0.2 m glycine). After blocking, the blot was incubated for 1 h with specific antibodies, followed by the ECL Plus Western Blotting Detection System (Amersham Biosciences). Anti-CSB (E18) and anti-Top1 (C21) antibodies were purchased from Santa Cruz Biotechnology. Anti-beta actin antibody was from Sigma.

**siRNA-mediated knockdown of CSB and CPFP mRNA**

siRNAs were designed to knockdown CSB and CPFP mRNAs (Table S2). Negative control siRNA (siLucGL3) was purchased from NIPPON GENE Co., Ltd. Transfection of siRNAs into HeLa cells was carried out by Lipofectamine 2000 (Invitrogen), whereas transfection into primary fibroblast cells or hTERT immortalized fibroblast cells was carried out by electroporation using Gene Pulser Xcell System (Bio-Rad).

**Purification of CSB protein complexes and co-immunoprecipitations**

HEK293 cells stably expressing the C-terminally FLAG-6×His-tagged WT-CSB, CPFP, 3PV-CSB (WT-CSB-HF, CPFP-HF and 3PV-CSB-HF) and N-terminally HA-FLAG-tagged Top1 (HAF-Top1) were established, respectively by using a pcDNA5/FRT Complete Kit (Invitrogen). The protein complexes were affinity-purified with nickel-NTA agarose (QIAGEN) and anti-FLAG M2-conjugated agarose (Sigma) from whole-cell extracts of HEK293 cells expressing WT-CSB-HF, CPFP-HF, and 3PV-CSB-HF, respectively. Whole-cell extracts of HAF-Top1 were immunoprecipitated with anti-FLAG M2-conjugated agarose. The bound proteins were eluted with the FLAG peptide and subjected to SDS–PAGE and immunoblotting.

**Transfection of CSB cDNA into hTERT-immortalized fibroblasts**

The C-terminally HA-FLAG-tagged cDNA of WT-CSB were cloned into the expression vector pCAGGS and transfected twice into an hTERT-immortalized CS-B fibroblast cell line TelCOFS03MA cells using Gene Pulser Xcell System (Bio-Rad). Twenty-four hours after the secondary transfection, cells were collected and analyzed by SDS–PAGE, Western blotting, and ICE assay.
Detection of Top1-cc by immunocomplex of enzyme (ICE) assay

ICE assay was performed as described previously (Muller et al. 1985; Miao et al. 2006) with small modifications. Cells (2 × 10⁶) were treated with camptothecin (CPT) (Sigma), lysed in lysis buffer (1% Sarkosyl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA) and homogenized with a 21G needle. The cell lysates were centrifuged on cesium chloride (CsCl) step gradients at 165 000 × g at 25 °C for 20 h. Then, 0.5 mL fractions were collected, and DNA peak fractions were quantified and applied to nitrocellulose membranes using a Minifold II Slot-Blot System (Whatman). Top1-DNA covalent complex (Top1-cc) was detected by Western blotting using anti-Top1 monoclonal antibody (C21) (Santa Cruz Biotechnology). The Slides were immersed in lysis and alkaline solution (125 mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol) immediately. These samples were analyzed and Top1 (C21) purchased from Santa Cruz Biotechnology. The slides were processed for alkaline electrophoresis. Slides were stained with SYBR Green I. Average tail moments of the comets from 83 to 91 cells per sample were obtained using Comet analyzer software (YOUWORKS).

Confocal imaging of EGFP-Top1, CSB-DsRed, and CPFP-DsRed

CS3AM primary fibroblasts were co-transfected with EGFP-Top1 plus CSB-DsRed constructs or EGFP-Top1 plus CPFP-DsRed constructs using Gene Pulser Xcell System (Bio-Rad). Twenty-four hours after transfection, cells were washed, fixed, and imaged using a Bio-Rad MRC1024 confocal laser scanning microscope (BioRad).

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RNA synthesis in CS-B cells after treatment with camptothecin

CPFP-knockdown and control TelCOFS02MA cells were treated with 20 μM CPT for 6 h and washed two times with PBS. After 0, 2, 3, 4, 6, 8, 16, and 24 h of incubation, cells were pulse-labeled for 1 h in DMEM containing [3H]-uridine (10 μCi/mL, GE Healthcare) to measure the RNA synthesis. Quantification of RNA synthesis was carried out as described previously (Horibata et al. 2004).

Camptothecin-induced degradation of Top1

CPFP-knockdown and control TelCOFS02MA cells were treated with CPT (2 μM) for the indicated times, or cells were treated with proteasome inhibitor MG-132 (5 μM) for 2 h before the addition of CPT (2 μM) plus MG-132 (5 μM). MG-132 was purchased from BIOMOL. After treatment of the cells with CPT or CPT plus MG-132, cells (1.5 × 10⁶) were washed twice with PBS and lysed in 200 μL of 2xSDS buffer (125 mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptopethanol) immediately. These samples were analyzed by Western blotting using antibodies against RNApoly 2A-10 and Top1 (C21) purchased from Santa Cruz Biotechnology.

Alkaline comet assay

Single-strand DNA breaks were quantified by the alkaline comet assay using Comet assay Kit (Trevigen) according to the manufacturer’s instructions. Cells were treated with CPT (2.5 μM) for 1 h in DMEM, washed twice with 1 × PBS (−), added with DMEM and incubated as indicated time for repair. At each time point, cells were trypsinized, and 2 × 10⁵ per mL cells were suspended in 1 × PBS. Then the cells were combined with molten LMAagarose and pipetted onto Comet-Slide. The Slides were immersed in lysis and alkaline solution and then processed for alkaline electrophoresis. Slides were stained with SYBR Green I. Average tail moment of the comets from 83 to 91 cells per sample were obtained using Comet analyzer software (YOUWORKS).

Confocal imaging of EGFP-Top1, CSB-DsRed, and CPFP-DsRed

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 RT-PCR/RFLP analysis of CSB mutation in CS3AM cells.

Figure S2 Western blot analysis of CPFP with anti-CSB and anti-PGBD3 antibodies in wild type, UV'1KO, TelCOFS02MA cells, and CPFP knockdown TelCOFS02MA cells.

Figure S3 Purification of WT-CSB, CPFP, 3PV-CSB and Top1 proteins.

Figure S4 Increased amount of CPT-induced Top1–DNA covalent complexes in the truncated CSB-expressing cells.

Figure S5 Anti-Top1 antibody (C21) recognized C-terminal region of Top1.

Figure S6 CPT-induced Top1-cc in CS-A cells.

Table S1 Summary of the CSB gene-related disorders
Table S2 Primers and siRNAs used in this study
Data S1 Materials and Methods.

Additional Supporting Information may be found in the online version of this article.

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