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DNA Repair Activity of Human rpS3 is Operative to Genotoxic Damage in Bacteria

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Abstract Human ribosomal protein S3 (rpS3), which has a DNA repair endonuclease activity, is a multifunctional protein. This protein is involved in DNA repair, translation, and apoptosis. In particular, rpS3 has a lyase activity, which cleaves the phosphodiester bond of damaged sites such as cyclobutane pyrimidine dimers and AP sites. Here, using deletion analysis, we identified that the repair endonuclease domain resides in the C-terminal region (165–243 aa) of rpS3. We also found that ectopic expression of GST-rpS3 in bacterial strain BL21 promoted the resistance of these cells to ultraviolet (UV) radiation and hydrogen peroxide (H_2O_2) treatment. The repair domain of rpS3 was sufficient to exhibit the resistance to UV irradiation and recover cell growth and viability, showing that the repair activity of rpS3 is responsible for the resistance to UV irradiation. Our study suggests that rpS3 is able to process DNA damage in bacteria via its repair domain, showing the resistance to genotoxic stress. This implies that rpS3-like activity could be operative in bacteria.

Key words: rpS3, DNA repair, repair domain, endonuclease, UV resistance

Ultraviolet (UV) light is divided into three parts [UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm)] and generates DNA damage in cells. Two of the most abundant mutagenic and cytotoxic DNA lesions are cyclobutylpyrimidone dimers (CPD) and 6-4 cyclobutyl-pyrimidone photoproducts (6-4 PP), and these lesions can be removed by a versatile repair mechanism [1]. The failure of these processes results in severe genomic instability that can lead cells to eventually becoming deleteriously cancerous.

The DNA repair system can be classified into several groups by its distinct mechanisms, such as mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), DNA double-strand break (DSB), and direct damage reversal repair [5, 9]. BER removes DNA-damaged bases, including oxidized DNA bases, alkylated DNA, incorporated uracil, fragmented pyrimidines, 8-oxo-7,8-dihydroguanine (8-OxoG), and thymine glycol, by DNA glycosylases. AP sites are also repaired by BER. NER removes bulky DNA adducts, such as UV-light-induced photolesions [(6-4) photoproducts (6-4PPs) and cyclobutane pyrimidine (cyclobutylpyrimidone) dimers (CPDs)], intrastrand cross-links, and large chemical adducts generated from exposure to genotoxic agents [29]. NER divides into two distinct pathways, termed global genomic repair (GGR) and transcription-coupled repair (TCR). While GGR is thought to be transcription-independent and removes lesions from the whole genome, TCR removes RNA-polymerase-blocking lesions from the transcribed strand of active genes [8, 14].

It was observed that several ribosomal proteins play roles in the translational apparatus and other extraribosomal functions [25], including the induction of apoptosis [26, 27], the suppression of tumors [38], the regulation of development [33], and DNA repair [12, 15, 16, 18, 19, 23, 24]. It has also been known that mammalian rpS3 functions as a DNA repair endonuclease and ribosomal protein S3.

Defects in the human DNA repair system have been shown to be associated with the photosensitive form of the human disorders such as xeroderma pigmentosum (XP) and trichothiodystrophy (TTD). These reports illustrate that DNA repair proteins increase resistance to DNA damage and confer an intrinsic genetic instability. There are 3 "UV endonuclease activities" in mammalian cells that cleave UV-irradiated DNA [18]. UV endonucleases are DNA excision repair enzymes that repair DNA damaged by UV-irradiation. They either remove the damaged base by DNA glycosylase activity to form an AP (apurinic/apyrimidinic) site and then excise the phosphodiester bond at the AP site by AP endonuclease activity, or have an excision endonuclease

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activity to recognize and excise bulky damaged sites [18, 19]. Interestingly, mammalian UV endonuclease III, rpS3, has a lyase activity on AP sites, and this protein is somehow missing in XP-D cell lines [19]. It is peculiar in that it also cleaves the phosphodiester bond within a cyclobutane pyrimidine dimer and has 100% sequence identity with ribosomal protein S3 [19]. *Drosophila* rpS3 also has repair activities on 8-oxoguanine and AP sites [12, 35], has a DNA deoxyribophosphodiesterase activity [28], and a DNA glycosylase activity [6]. RpS3 is known to be crosslinked to eukaryotic initiation factors, eIF-2 and eIF-3, and appears to be directly involved in ribosome-mRNA-aminoacyl tRNA interactions during protein synthesis [3]. Recently, it was proposed that human rpS3 is involved in apoptosis in lymphocytes [13]. Therefore, rpS3 seems to function in translation, DNA repair, and apoptosis.

In this study, we revealed that rpS3 has a functional repair domain in the C-terminus and renders the resistance to UV and hydrogen peroxide (H_2O_2) in *E. coli*. The repair domain of rpS3 was sufficient for UV resistance mediated by the protein. Thus, the resistance to DNA damage derived from rpS3 was in concordance with the repair activity caused by a repair domain in rpS3.

MATERIALS AND METHODS

Plasmids and Bacterial Strains

Human rpS3 and its mutants were subcloned into pGEX-5X-1 (Amersham) in frame with GST, generating GST-rpS3 and various deletion mutants that were made by PCR fused with GST fusion genes. The DNA repair deficient strain BW528 (deficient in exonuclease III [*xth*] and endonuclease IV [*nfo*]) was a gift from Dr. Bernard Weiss (EMORY University, Atlanta, U.S.A.).

Antibodies and Immunoblot Analysis

Antibodies against GST were purchased (Santa Cruz, U.S.A.). Immunoblotting was performed using chemiluminescence blotting substrate (Boehringer Mannheim, Germany). Horseradish peroxidase conjugated anti-rabbit IgG (Boehringer Mannheim) was used as secondary antibodies.

Overexpression of GST Fusion Constructs and Purification

BL21pLysS bacterial strain transformed with GST-rpS3 or mutants were cultured overnight at 37°C, diluted in LB+ ampicillin (Amersham) media to an OD_{600}=0.1, and grown at 37°C until cultures reached at OD_{600}=0.7. Then, isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Gibco BRL) was added to a final concentration of 0.5 mM, and growth was continued for 3 h at 30°C. Cells were pelleted by centrifugation and resuspended in lysis buffer [40 mM Tris-HCl (pH 8.0), 70 mM KCl, 0.01% Triton X-100, 3 mM EDTA, 10 mM 2-mercaptoethanol] containing protease inhibitors (1 μg/ml pepstatin, 1 μg/ml PMSF, 5 μg/ml leupeptin, 10 μg/ml aprotinin). Cells were sonicated twice, 15 s each time with 70% pulse, and debris were removed by centrifugation. The supernatant was then applied to a Glutathione-Sepharose (Amersham) affinity column, washed three times with 1×PBS, and the fusion construct was eluted with 10 mM reduced glutathione (50 mM TrisHCl, pH 7.5).

DNA Repair Assay of rpS3.

UV-specific DNA repair endonuclease assay was performed in reaction buffer [40 mM Tris-HCl (pH 8.0), 70 mM KCl, 0.01% Triton X-100, 3 mM EDTA, 10 mM 2-mercaptoethanol] containing 2.5 nmol of UV-irradiated PM2 ^{3}H-labeled DNA, as described previousely [15, 16, 19]. One unit of enzyme defines 1 fmol nick at 37°C for 1 min. Wild-type GST-rpS3 or deletion mutants expressed in bacteria were purified by GST beads (Amersham) and monitored for endonuclease activity. The reaction mixture was filtered with nitrocellulose filters (Bio-Rad), and these filters were dried and counted by liquid scintillation (Beckman).

Measurement of UV Resistance

BL21pLysS and BW528 bacterial strains transformed with GST-rpS3 or mutants were cultured overnight at 37°C, diluted in LB+ampicillin media to an OD_{600}=0.1, and grown at 37°C until cultures reached an OD_{600}=0.7. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Gibco BRL) was added to a final concentration of 0.5 mM, and growth continued for 2 h at 30°C. Cells were diluted in 100 μl of medium to an OD_{600}=0.25, exposed to 20 or 200°J/m^2 UV irradiation by CL-1000 Ultraviolet crosslinker (UVP), seeded in 2.5 ml of medium, and cultured at 37°C. For hydrogen peroxide assay, cells were diluted in 2.5 ml of medium to an OD_{600}=0.1 and treated with 0.5 mM hydrogen peroxide (Fluka). Cell growth was measured at OD_{600} by spectrophotometery (Shimadzu). For measurement of survival rate under UV irradiation, cells were diluted to $1:10^{-6}$–10^{-7} (BL21) or $1:10^{-3}$–10^{-4}, plated, and incubated for 16 h at 37°C. The number of colonies was counted.

RESULTS

RpS3 has a Functional Domain for DNA Repair

Similar to many other dual functional proteins, rpS3 appears to have a domain for DNA repair. To more precisely define the repair domain for the endonuclease activity of rpS3, various deletion mutants were constructed in GST vector, expressed in a bacterial expression system, and then purified by affinity columns (Fig. 1A). Using wild-type and deletion mutants of rpS3 fused with GST, *in vitro* nick circle UV-endonuclease assay was performed to determine the repair domain of rpS3. Surprisingly, a mutant (rpS3ΔN164; Δ1-

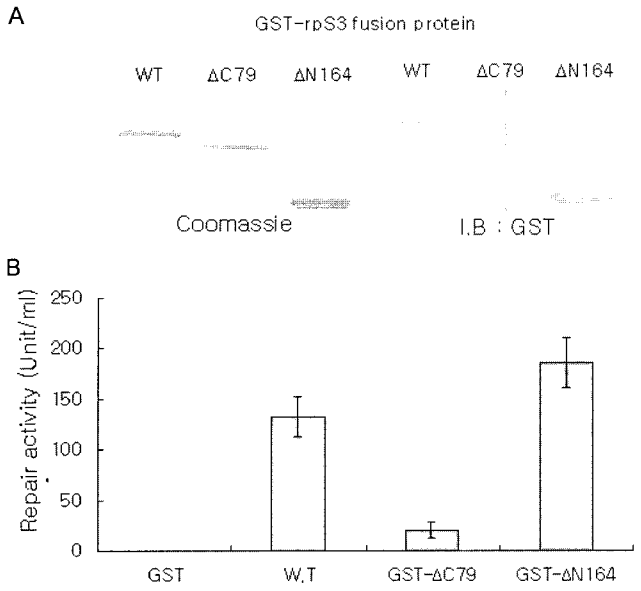

Fig. 1. RpS3 has a domain for DNA repair.
(A) GST-rpS3, GST-rpS3ΔC79, and GST-rpS3ΔN164 proteins were expressed in bacteria, purified, resolved by 12% SDS-PAGE, and stained by Coomassie or immunoblotted (IB) by using anti-GST antibodies. (B) GST-rpS3, GST-rpS3ΔC79, and GST-rpS3ΔN164 proteins were expressed in bacteria and purified, and 0.1 μg each of proteins was monitored by *in vitro* DNA repair assay.

164) deleted with most of the N-terminus still had its repair activity, even though it contained only 79 amino acids from the C-terminus. However, deletion of these 79 amino acids from the C-terminus (rpS3ΔC79) showed disruption of the repair activity (Fig. 1B), indicating that the repair domain resides in the C-terminus.

RpS3 Overcomes the Toxicity of UV Irradiation

To study the direct role of UV-damage-dependent repair enzyme in cells, GST, GST-rpS3, GST-rpS3ΔN164, or GST-rpS3ΔC79 were transformed into bacteria BL21 or BW528. The *E. coli* strain BW528, which is deficient in repair enzymes such as exonuclease III (*xth*) and endonuclease IV (*nfo*), is known to be sensitive to oxidative agent and radiation [4, 10]. As seen in Fig. 2A, the growth of BW528 was slightly slower than normal bacterial strain BL21. While GST-rpS3ΔC79 showed a growth inhibitory effect in both strains, GST-rpS3 slightly increased the growth of BW528 (Figs. 2B, 2C). However, the growth of BL21 cells transformed with GST-rpS3 was slightly slower than the cells transformed with GST (Fig. 2C).

We next analyzed the effect of GST only and GST-rpS3 under UV irradiation in both strains. One hour after exposure to 20 J/m^2 of UV, growth of BL21 strain cells transformed with GST decreased to significant levels in a dose-dependent manner (Fig. 3). To confirm the effect of rpS3 for the resistance of cells against UV irradiation, BW528 or BL21 bacterial strains cells transformed with GST-rpS3 were

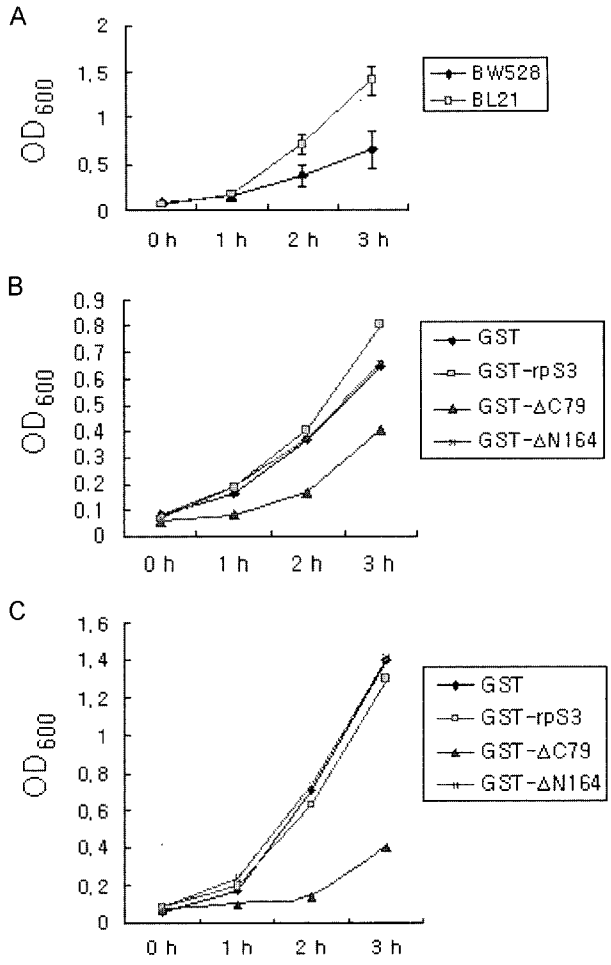

Fig. 2. Growth of BW528 and BL21 strains.
(A) BW528 or BL21 cells were diluted in 2.5 ml medium to an OD_{600}=0.1, and cell growth was spectrophotometrically measured at OD_{600}. Values are represented by means of three independent experiments. (B) GST, GST-rpS3, GST-rpS3ΔN164, or GST-rpS3ΔC79 were transformed into bacteria BW528, and the growth curve of each culture was determined with a spectrophotometer at OD_{600}. (C) GST, GST-rpS3, GST-rpS3ΔN164, or GST-rpS3ΔC79 were transformed into bacteria BL21, and growth of each culture was spectrophotometrically measured at OD_{600}.

exposed to UV and measured for their growth rate. In BW528 strain, the growth rate of cells transformed with GST-rpS3 was faster than that of cells transformed with GST, but did not show a significantly improved recovery rate under UV irradiation (Fig. 4A), implying that the normal repair system is necessary for rpS3-like repair activity in bacteria. In contrast, the growth rate of cells transformed with GST-rpS3 or GST was similar, but the recovery rate in BL21 strains was significantly increased by GST-rpS3 under UV irradiation (Fig. 4B). This indicates that rpS3 overcomes the toxicity of UV by means of DNA repair processing and restores the growth rate of damaged cells. This effect also appeared in BL21 strains at a low dose of UV (Fig. 4C).

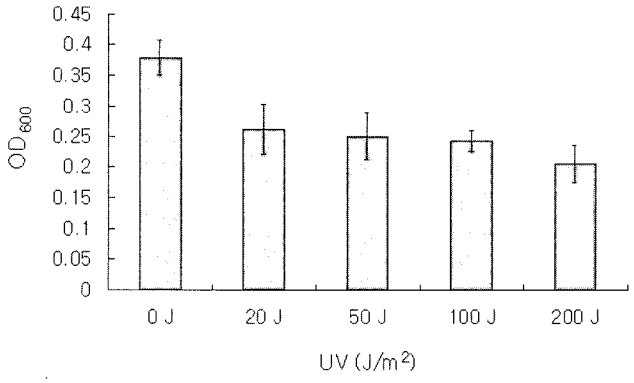

Fig. 3. Toxicity of UV radiation to GST-expressing BL21 cells. BL21 cells containing the GST control vector were irradiated with UV. One hour after irradiation with various doses of UV, cell numbers were determined by spectrophotometery. Values are represented by means of three independent experiments.

Repair Domain of rpS3 is Sufficient for UV Resistance

To investigate the potential physiological consequences of the repair activity of rpS3 under UV, we studied the growth rate of cells transformed with the repair domain of rpS3. As shown in Figs. 5A and 5B, a similar resistance to UV was shown in cells transformed with the repair domain of rpS3, suggesting that UV resistance via rpS3 is arisen from the repair activity of rpS3. The results suggest that human rpS3 repairs damaged DNA in UV-irradiated bacterial cells and recovers their growth rate efficiently.

RpS3 Increases the Survival Rate Under UV Irradiation

The ability of rpS3 to protect DNA against UV-irradiation-induced damage was also quantitatively analyzed by counting the colonies. After UV irradiation, cells were plated and the survival rate was determined. As expected, both BW528 and BL21 strains transformed with the repair domain of rpS3 showed cell viability higher than those transformed with GST alone (Figs. 6A, 6B). Thus, the repair domain of rpS3 appears to be sufficient for this complementation, indicating that the increased viability by rpS3 is resulted from the repair activity.

RpS3 also Overcomes H_2O_2-Induced DNA Damage

Generation of reactive oxygen species (ROS) in response to oxidative stress induces damage to DNA, lipids, and proteins [2, 24, 32]. The formation of DNA strand breaks (DNA-SB) is among the earliest abnormalities that occur within cells exposed to oxidative stress. Therefore, we next analyzed whether rpS3 overcomes growth rate under oxidative stress that induces DNA damage. Interestingly, cells transformed with the repair domain of rpS3 showed higher growth rate than those transformed with GST alone under the treatment of hydrogen peroxide (H_2O_2) (Fig. 7A), indicating that rpS3 functions against oxidative stress through its repair activity and recovers the abnormality of damaged cells.

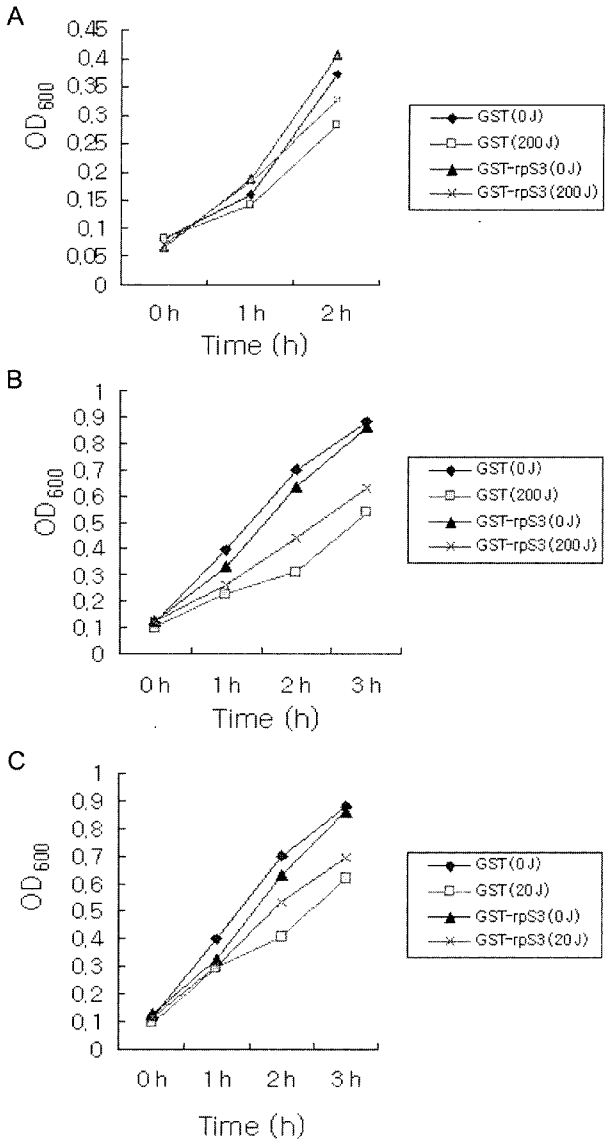

Fig. 4. RpS3 is responsible for increased UV resistance. (A) BW528 cells transformed with GST alone or GST-rpS3 were exposed to 200 J/m² of UV, and cell numbers were determined by spectrophotometery at various times after irradiation. (B) BL21 cells transformed with GST alone or GST-rpS3 were exposed to 200 J/m² of UV, and cell numbers were determined by spectrophotometery at various times after irradiation. (C) BL21 cells transformed with GST alone or GST-rpS3 were exposed to 20 J/m² of UV, and cell numbers were determined by spectrophotometery at various times after irradiation. Experimental points are means of three independent experiments.

DISCUSSION

DNA repair is the major mechanism to protect damaged cells from abnormality. In addition, cell-cycle arrest in response to DNA damage gives cells the time to repair the damage [1, 17]. These pathways are modulated to maximize cellular survival while minimizing the chance of

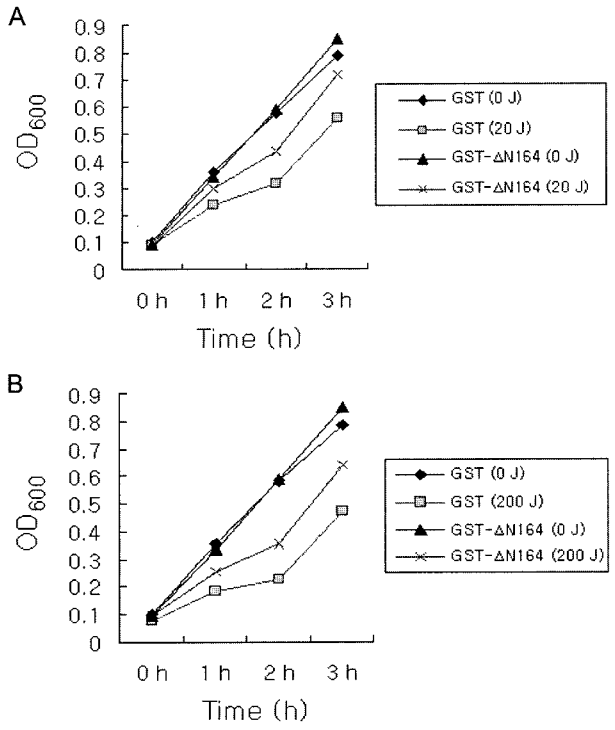

Fig. 5. Repair domain of rpS3 is sufficient for UV resistance.
(A) BL21 cells transformed with GST alone or GST-rpS3ΔN164 were exposed to 20 J/m^2 of UV, and growth rate was measured by spectrophotometrically counting cells at the indicated time points after irradiation. (B) BL21 cells transformed with GST alone or GST-rpSΔ3N164 were exposed to 200 J/m^2 of UV, and growth rate was measured by spectrophotometrically counting OD of cells at the indicated time points after irradiation. All points are means of three independent experiments.

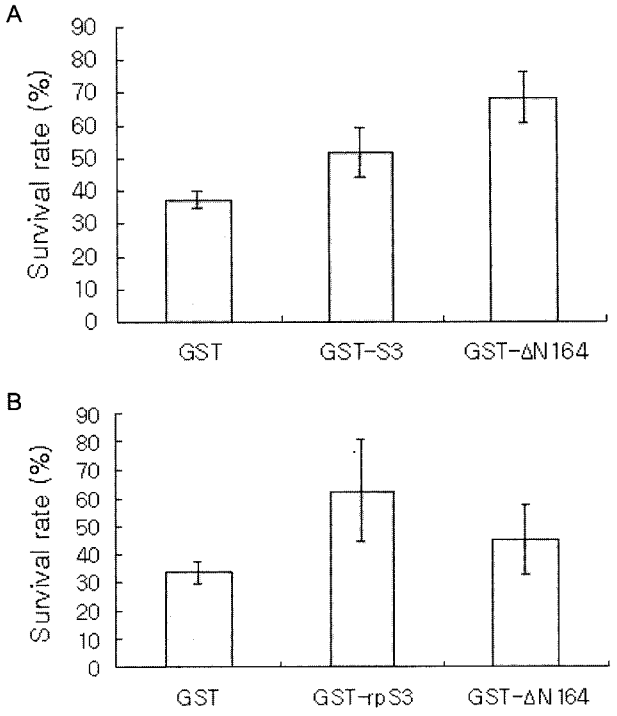

Fig. 6. RpS3 increases the survival rate after UV irradiation.
(A) BW528 cells transformed with GST alone, GST-rpS3, or GST-rpS3ΔN164 were exposed to 200 J/m^2 of UV, plated, and incubated at 37°C. After 16 h, the number of colonies was counted. The survival ratio is the ratio of the percent survival between UV-irradiated cells and non-irradiated cells. (B) BL21 cells transformed with GST alone, GST-rpS3, or GST-rpS3ΔN164 were exposed to 200 J/m^2 of UV, plated, and incubated for 16 h at 37°C and the number of colonies was counted. All points are means of three independent experiments.

carcinogenesis. It was of interest, therefore, to study the capability of rpS3 through the expression of the protein and to examine whether it alone as the first step is sufficient to rescue DNA-damaged cells *in vivo*. For this purpose, we used bacteria transformed with rpS3 to test if this step was also operative in *E. coli*.

UV radiation is a very prominent environmental toxic agent, and conjugated bonds in organic molecules absorb short wave of UV radiation around 200 nm [37]: Proteins that contain tryptophan or tyrosine can, therefore, absorb solar UV radiation. Thus, UV exposure may cause protein damage and disturbances in signaling pathways. All the bases in DNA contain an abundance of conjugated bonds in ring structures, making DNA a target of damage by UV radiation in cells. Therefore, the genome in cells is easily damaged upon UV irradiation, and mutations may subsequently occur. RpS3 is known to be a UV DNA repair endonuclease that has a lyase activity on AP and UV-irradiated DNA, and repairs the UV-damaged DNA [19]. Our data suggest that rpS3 acts to remove damaged DNA and recover growth in UV-irradiated cells (Fig. 4).

Oxidative stress also attacks DNA and causes oxidative damage to bases and the sugar-phosphates, as well as single- or double-strand breaks [1, 2, 17, 24, 32]. Base damage is caused by reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical (OH·), and hydrogen peroxide (H_2O_2). Cells remove ROS by superoxide dismutase, catalase, and hydrolyase to defend against oxidative stress. Another defense mechanism is the DNA repair processes, including base excision repair (BER), transcription-coupled repair (TCR), global genome repair (GGR), mismatch repair (MMR), homologous recombination (HR), and non-homologous end-joining (NHEJ). BER is the major pathway for repair of oxidative base damage, and the key enzymes involved in this process are DNA glycosylases, AP-endonucleases, AP-lyases, DNA polymerases, and DNA ligases [30]. RpS3 has an AP-lyase activity [18, 19], indicating that the recovery of growth rate in GST-rpS3-transformed cells arose from the increased repair rate of BER by rpS3 (Fig. 5A).

In *Escherichia coli*, several complicated DNA repair systems preventing mutagenesis on damaged DNA have been found. The GO (7,8-dihydro-8-oxoguanine) system consists of MutT (8-oxo-dGTPase), MutM (2,6-dihydroxy-5N-formamidopyrimidine (Fapy)-DNA glycosylase, Fpg),

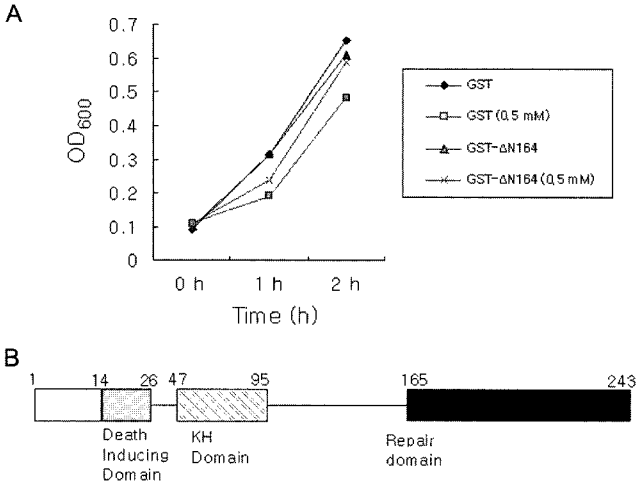

Fig. 7. Oxidative stress and rpS3.
(A) BL21 cells transformed with GST alone or GST-rpS3ΔN164 were treated with 0.5 mM H_2O_2, as described in Materials and Methods. Cell growth rate was measured by spectrophotometery at the indicated time points after the treatment (average of three experiments). (B) Schematic representation of human rpS3. The KH domain is a highly conserved motif, usually found in RNA-binding proteins.

and MutY (adenine-DNA glycosylase). The mismatch repair (MMR) requires the MutS, MutL, MutH, and DNA adenine methylase (Dam) proteins [22]. Moreover, nucleotide excision repair involves the UvrABC complex, that forms the UvrB-DNA complex, and subsequent DNA incisions on either side of the damage by UvrC [7]. In these systems, endonucleases such as *E. coli* endonuclease III and UvrC play an important role in the removal of the sugar phosphate residues on the 3'-end in the repair process. In the present study, human rpS3 was found to invigorate DNA repair under UV irradiation and oxidative stress. Taken together, our results strongly indicate that human rpS3 could supplement the repair system in *E. coli* as an endonuclease.

Based on previous studies, rpS3 appears to have structural similarities with the *E. coli* repair proteins endonuclease III, MutY, and AlkA 3-methyladenine DNA glycosylase [21, 31]. In *E. coli* endonuclease III, it was proposed that the Glu-112 residue was responsible for the N-glycosylase mechanism and the Lys-120 residue for the β-elimination mechanism [21]. Furthermore, both Lys120 and Asp138 seemed to be catalytically important, lying at the mouth of the active pocket. In contrast, the Lys-191 residue is known as the DNA-binding residue within the active site pocket [31]. In *Drosophila*, the Gln-59 residue, which is absent in human rpS3, plays an important role for N-glycosylase activity. This difference explains that human rpS3 lacks the glycosylase ability to remove 8-oxoG, whereas *Drosophila* S3 possesses the exceptional ability to remove this lesion [11]. In this study, the C-terminal region of rpS3 seems to be sufficient for the β-elimination reaction without the important residues at the mouth of the active pocket, suggesting that the DNA-binding residue seems to reside in the C-terminal region in human rpS3 (Fig. 7B).

It has been shown that rpS3 induces apoptosis in lymphocytes [23]. We recently showed that human rpS3 repairs various DNA damage *in vitro* [20]. In the present study, we found the ability of rpS3 to repair damaged DNA and recover growth rate in *E. coli*. It is to be noted that rpS3 increases the survival of *E. coli*, probably also in mammals, and that it induces apoptosis in the well-described apoptotic system such as lymphocytes, probably also in certain circumstances. These are the subjects of our ongoing study [23].

In summary, we have shown that rpS3 has a repair domain for an endonuclease activity and offers protection against the production of UV- and H_2O_2-induced DNA-damage break, suggesting that rpS3-like activity might be operative in *E. coli*.

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