RpS3, a DNA repair endonuclease and ribosomal protein, is involved in apoptosis

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Abstract **It is known that mammalian rpS3 functions as a DNA repair endonuclease and ribosomal protein S3. It was also observed that several ribosomal proteins or DNA repair enzymes are related to apoptosis. We report here a third function of rpS3, induction of apoptosis. The localization of green fluorescent protein (GFP)-rpS3 is changed to the nuclear membrane when lymphocytic cells undergo rpS3-induced apoptosis. Transient expression of GFP-rpS3 activates caspase-8/caspase-3 and sensitizes cytokine-induced apoptosis. Deletion analysis reveals that the two functions of rpS3, DNA repair and apoptosis, use independent functional domains.**

Key words: rpS3; DNA repair; Apoptosis; Cytokine; Lymphocyte

1. Introduction

Mammalian cells respond to DNA damage signals by activating cell cycle checkpoints which arrest the cell cycle, and activating DNA repair systems, or inducing apoptosis. It is well known that there must be a checkpoint regulator for DNA repair and apoptosis during DNA damage responses [1]. Apoptosis is characterized by distinct morphological and biochemical changes that take place upon the activation of a family of caspases, which require proteolytic processing upon initiation of apoptosis [2]. Two major signaling pathways, the mitochondria-dependent pathway and the mitochondria-independent pathway, result in the activation of caspases [3,4]. In the instructive apoptosis, death receptors that transmit apoptosis signals by specific death ligands play a central role. These receptors can activate death caspases through the intracellular death domain. Trimerization of these receptors is critical for recruitment of adapter molecules to initiate caspase activation [5].

The ribosome is a large ribonucleoprotein machine that synthesizes proteins from transcribed mRNA. It is well known that ribosomes consist of a large and a small subunit and the small subunit mediates the interactions between mRNA and tRNAs [6]. Several ribosomal proteins have also been found to play roles in the translational apparatus and other extra-ribosomal functions [7], including induction of apoptosis [8,9], suppression of tumors [10], regulation of development [11], and DNA repair [12–17].

Interestingly, mammalian UV DNA repair endonuclease III has a lyase activity, cleaves the phosphodiester bond within a cyclobutane pyrimidine dimer and AP site, and has 100% sequence identity with rpS3 [12]. *Drosophila* rpS3 has an AP lyase activity on abasic site DNAs [18,19], a DNA deoxyribophosphodiesterase activity [20], and a DNA glycosylase activity on 8-oxoguanine [21]. RpS3 forms a part of the domain on the ribosome where the initiation of translation occurs; it can be cross-linked to the eukaryotic initiation factors eIF-2 and eIF-3, and appears to be directly involved in the ribosome–mRNA–aminoacyl tRNA interactions during protein synthesis while locating in the decoding center [22]. Therefore, rpS3 seems to function both in translation and in DNA repair.

In this study, we demonstrate for the first time that rpS3 induces apoptosis whose signal is executed through the activation of caspase-8 followed by caspase-3 activation. More importantly, this protein increases the proapoptotic potential of cytokines. These data suggest that the DNA repair pathway and apoptosis pathway might cross-talk via rpS3.

2. Materials and methods

2.1. Plasmids

RpS3 and its mutants were subcloned into pEGFP-C1 vector (Clontech) in frame with GFP (green fluorescence protein), generating GFP-rpS3 and various deletion mutants fused with GFP fusion genes.

2.2. Cell culture and transfection

MPC-11 (mouse plasmacytoma) B cells were cultured on 10 cm plates in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Gibco BRL). Jurkat human T cells were cultured in RPMI, supplemented with 10% fetal bovine serum (Gibco BRL). Cells were transfected using Superfect Transfection Reagent (Qiagen), and lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitors (1 μg/ml pepstatin, 1 μg/ml phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 10 μg/ml aprotinin) and sonicated briefly, followed by immunoblotting.

2.3. Antibodies and immunoblot analysis

Monoclonal antibodies against GFP epitope (Roche), caspase-3 (Santa Cruz Biotechnology) and caspase-8 (Upstate Biotechnology) were used for apoptotic activity of rpS3. Polyclonal antibodies against human rpS3 were purchased from BioInstitute, Korea University.

Cell extracts were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitors (1 μg/ml pepstatin, 1 μg/ml PMSF, 5 μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were sonicated briefly and cleared by centrifugation. Proteins from total cell lysate were electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gel electro-

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phoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Bio-Rad). Immunoblotting was performed using chemiluminescence blotting substrate (Roche). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (Roche) were used as secondary antibodies.

For subcellular fractionation, cells were harvested, washed with phosphate-buffered saline (PBS) and taken up in buffer I containing 10 mM HEPES (pH 7.9), 10 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and protease inhibitors. After incubation on ice for 15 min, plasma membranes were disrupted by adding 0.3% Nonidet P-40 and mixed for 10 s. The nuclei were pelleted by centrifugation at 13 000 rpm for 1 min, and a cytoplasmic fraction (supernatant) was recovered. After washing three times, the pellet was resuspended in buffer II (20 mM HEPES [pH 7.9], 0.5 mM EDTA, 0.5 mM EGTA, 0.4 M NaCl, 1 mM DTT, protease inhibitors). After rocking for 30 min on ice, the samples were centrifuged for 15 min at 4°C to recover a nuclear fraction.

2.4. Detection of apoptosis

Apoptosis was detected by DNA fragmentation, fluorescence-activated cell sorting (FACS), and nuclear morphology assays. For FACS analysis, detached cells were pooled and fixed overnight with 75% ethanol in PBS at 4°C. After rinsing three times with PBS, cells were incubated for 30 min with 1 ml of PBS containing 0.1 mg of RNase and 50 μg of propidium iodide. A total of 1×10^4 cells were then analyzed in a flow cytometer (FACSCalibur; Becton Dickinson).

For microscopic observation of apoptotic cells, pEGFP-C1 and other GFP fusion vectors were transiently transfected into MPC-11 cells in 6 cm plates using Superfect (Qiagen). Twenty-eight hours after transfection, image was collected using a fluorescence microscope (Zeiss).

For DNA fragmentation assays, cells were transfected using Superfect transfection reagent (Qiagen), and lysed using lysis buffer (5 mM Tris–HCl [pH 8.0], 20 mM EDTA, 0.5% Triton X-100) and digestion buffer (100 mM NaCl, 10 mM Tris–HCl [pH 8.0], 24 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K). The DNA in the lysate was precipitated with 2 volumes of 100% ethanol and 1 M ammonium acetate. The DNA precipitate was resuspended in 30 μl of TE (pH 8.0, 200 μg/ml DNase-free RNase A). After incubation at 37°C for 1 h, the DNA was loaded onto a 2% agarose gel and electrophoresis was conducted at 50 V for 4 h in 1×Tris-acetate-EDTA (TAE) buffer. The gel was stained with 2 μg/ml ethidium bromide for 15 min, destained with water for 1 h, and visualized under UV light.

3. Results

3.1. RpS3 is involved in apoptosis

DNA damage signals trigger two pathways; one is related to the cell cycle checkpoint which arrests the cell cycle for DNA repair and the other is related to apoptosis to avoid carcinogenesis. Cells undergoing apoptosis show distinctive morphological changes, including membrane blebbing, cytoplasmic and nuclear condensation, loss of microvilli, and formation of apoptotic bodies [23]. Another biological hallmark of apoptosis is the cleavage of chromosomal DNA into nucleosomal fragments that is performed by caspase-activating DNase, a DNase which has an endonuclease function activated by caspase-3 [24]. Therefore it was of interest to study the relationship between repair endonuclease activity and apoptotic endonuclease activity. Surprisingly, it has been revealed that rpS3a, which is another ribosomal protein distinct from rpS3, has a role in the apoptotic process [8]. RpS3 might play a role in apoptosis either as a DNase or as a signal mediator between DNA repair and apoptosis.

To assess the apoptotic effect of rpS3, we performed DNA fragmentation analysis. RpS3 was transfected into MPC-11 cells by liposome-mediated transfection, and total cell lysates were prepared 26 h after transfection for the detection of nucleosomal fragments. As shown in Fig. 1A, DNA fragmentation analysis of MPC-11 lysates revealed that GFP-rpS3 induced fragmentation of chromosomes.

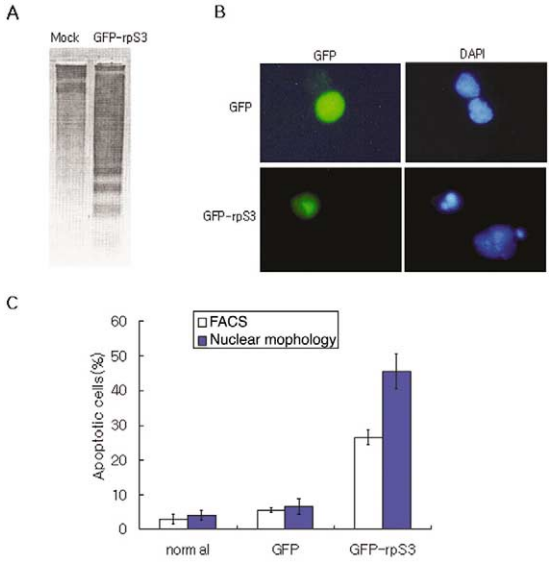

Fig. 1. RpS3 is directly involved in apoptosis. A: RpS3 constructed in pEGFP-C1 vector was expressed in MPC-11 cells for 26 h and then the lysate was resolved by 2% agarose gel. Transfection of the GFP expression vector (pEGFP-C1) containing GFP alone served as a negative control (mock). B: GFP-rpS3 was transfected into MPC-11 cells. Twenty-six hours after transfection, cells were fixed. Shown are representative non-apoptotic GFP cells and apoptotic GFP-rpS3 cells. Both GFP and GFP-rpS3 transfected cells were stained with DAPI to detect nuclear fragmentation or condensation. C: GFP and GFP-rpS3 were transfected to MPC-11 cells. After 26 h, the number of apoptotic cells was determined by flow cytometry or fluorescence microscopy.

It was also confirmed that the nuclei of cells over-expressing GFP-rpS3, which were stained with DAPI, were condensed or fragmented in the manner of apoptotic cells (Fig. 1B). Among green cells expressing GFP-rpS3, over 25–50% of the cells underwent apoptosis, while 5% of the cells expressing GFP were apoptotic (Fig. 1C). A similar apoptotic effect was obtained in human cell lines such as Jurkat but low in fibroblasts.

3.2. The localization of rpS3 under rpS3-induced apoptosis

Endogenous rpS3 is localized in the cytoplasm as a ribosomal protein, but also exists in the nuclei at a ratio of approximately 6:4 (Fig. 2A). To examine the change of subcellular localization of rpS3 proteins, cells were transfected with plasmids containing rpS3 (GFP-rpS3). In normal cells, the expression of GFP-rpS3 fusion protein was mostly found in the cytoplasm but a smaller amount is also found in the nuclei (Fig. 2B, upper panels). When cells undergo rpS3-induced apoptosis, GFP-rpS3 was translocalized towards the nuclear membrane (Fig. 2B, lower panels). Furthermore, the cells that expressed GFP-rpS3 in the nuclei but still maintained normal nuclei underwent apoptosis as time passed by (Fig. 2C), suggesting that translocation of GFP-rpS3 into nuclei is important in rpS3-induced apoptosis.

3.3. RpS3 activates caspase-8 and caspase-3 as downstream effectors

How is apoptosis triggered by rpS3? It is well established that the major downstream event of the apoptotic pathway is

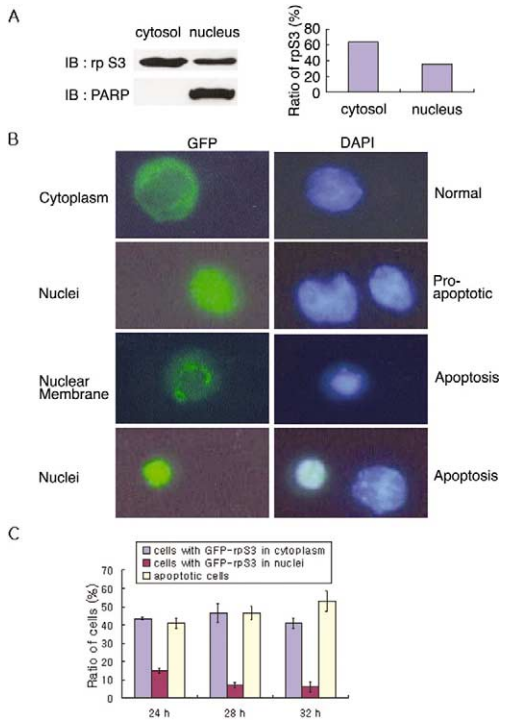

Fig. 2. Subcellular localization of rpS3 and apoptosis. A: MPC-11 cells were fractionated into cytosolic and nuclear fractions. Equal volumes of cytosolic and nuclear fractions were analyzed by 12% SDS–PAGE and immunoblotting with anti-rpS3 antibodies. Anti-poly(ADP-ribose) polymerase antibodies were used as a control. B: Twenty-six hours after transfection of GFP-rpS3, MPC-11 cells were fixed and detected by their green fluorescence (green). Changes of the chromatin structure in the cells were monitored by DAPI staining (blue). C: Jurkat cells were transfected with pEGFP-rpS3. At the time points indicated, cells were fixed and counted by fluorescence microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

composed of a caspase cascade which is initiated by the activation of caspase-8 followed by caspase-3 [25]. To elucidate the downstream events of rpS3-induced apoptosis, Jurkat cells were transfected with GFP-rpS3 then treated with z-DEVD-fmk and z-IETD-fmk, which are direct inhibitors of caspase-3 and caspase-8, respectively. We found that both inhibitors blocked rpS3-induced cell shrinkage, nuclear condensation and fragmentation (Fig. 3A), indicating that the activities of caspase-8 and caspase-3 are required for the apoptotic effect of rpS3 whose signal is transmitted from caspase-8 to caspase-3. Similar results were obtained when rpS3-transfected cell lysates were assayed with Ac-DEVD-AFC and Ac-IETD-AFC, which are substrates of caspase-3 and caspase-8, respectively (Fig. 3B).

Furthermore, immunoblotting with anti-caspase-8 antibody showed that the active form of endogenous caspase-8 was increased in rpS3-transfected cell lysates (Fig. 3C). In the case of caspase-3, rpS3 also increased the active form of caspase-3, suggesting that rpS3 acts upstream of the caspase-8/caspase-3 pathway.

3.4. *RpS3 is involved in the cytokine-mediated apoptosis pathway*

We then tested whether rpS3 is involved in the signal transduction of cytokine-mediated apoptosis which has been extensively investigated as a model of programmed cell death via caspases-8 and -3. Fas ligand and tumor necrosis factor α (TNFα) are known to trigger the downstream death signals by the trimerization of death receptors, inducing the association of death domains and the recruitment of Fas binding molecules including FADD/MORT1 and caspase-8 [26]. Therefore, we tested the cell death rates of MPC-11 cells with or without transient transfection of GFP-rpS3 upon the induction of apoptosis by Fas ligand or TNFα. GFP-rpS3 enhances apoptosis synergistically in the presence of cytokines (Fig. 4A), suggesting that rpS3-induced apoptosis helps the cytokine-induced apoptosis pathway and increases the sensitivity for cytokines. To further assess the localization of rpS3 under cytokine-induced apoptosis, we have fractionated cytosol or nucleus from cytokine-treated or non-treated cells. In contrast to the cytokine-untreated cells, endogenous rpS3 was translocated into the nuclei in cytokine-treated cells (Fig. 4B), resulting in a change of the normal distribution ratio of nuclear and cytoplasmic rpS3 proteins as shown in Fig. 2A. This indicates that the translocation of rpS3 around the nucleus is an important event in cytokine-induced apoptosis. It is to be noted that the translocation appears to be more

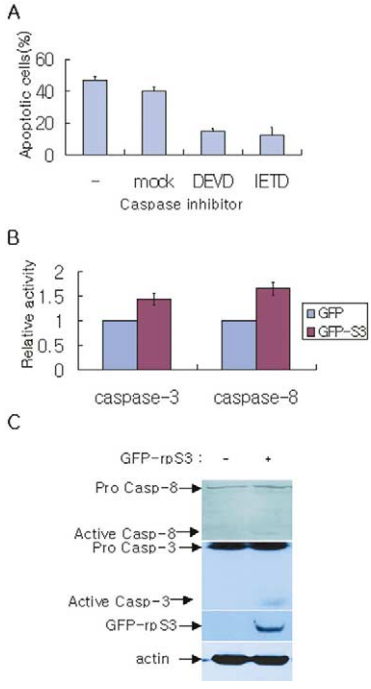

Fig. 3. RpS3 executes apoptosis via caspase-8 and caspase-3. A: Effect of the caspase inhibitors z-DEVD-fmk and z-IETD-fmk on the induction of apoptosis by rpS3. Jurkat cells were transfected with pEGFP-rpS3 (GFP-rpS3). Then 3 h after transfection, cells were treated with dimethylsulfoxide carrier (mock) or 20 μM/ml z-DEVD or z-IETD. After 25 h, the percentage of apoptosis was calculated by counting the cells with fragmented nuclei among transfected cells by fluorescence microscopy. B: Caspase activity in rpS3-induced apoptosis. Jurkat cells were transfected with pEGFP-rpS3. After 26 h, cleavage of fluorogenic Ac-DEVD-AMC and Ac-IETD-AMC by cell lysates was measured for 30 min using a fluorescent plate reader. C: Activation of procaspase-3 and procaspase-8. Following pEGFP-rpS3 transfection, Jurkat cells were collected and lysed. Lysates were examined by immunoblotting (IB) with anti-caspase-3 or anti-caspase-8 antibodies. The positions of the procaspase-3 and procaspase-8 precursors and active caspase-3 and caspase-8 are indicated as arrows.

significant in cells treated with TNFα than those with Fas ligand.

3.5. *RpS3 has distinct functional domains for DNA repair and apoptosis*

Like many other dual-function proteins, rpS3 may have independent domains for DNA repair and apoptosis. To confirm this prediction, various deletion mutants of rpS3 were constructed and apoptosis-inducing abilities were tested. Deletion constructs encoding GFP-fused proteins were transfected into MPC-11 cells to find the death-inducing domain in rpS3. Mutants with C-terminal deletions (ΔC66, ΔC217) that contain 177 and 26 amino acids respectively from the N-terminus are believed to be defective in endonuclease activity as reported previously [27,28]. However, these proteins showed a slightly increased apoptosis-inducing ability. Mutants with N-terminal deletions also showed increased apoptotic ability until K18 was deleted (Fig. 5A). It is to be noted that the domain with N-terminal 26 amino acids (ΔC217), which is distinct from the endonuclease domain of rpS3, appears to be sufficient to induce apoptosis. In contrast, the deletion mutant ΔN18 (Δ1–18 mutant) significantly reduced the death-inducing ability, suggesting that 15–26 amino acids of the N-terminus are critical for apoptosis. These results imply that rpS3 has two independent domains, a repair domain which resides in the C-terminus [18,27,28] and a death-inducing domain in the N-terminus (Fig. 5B).

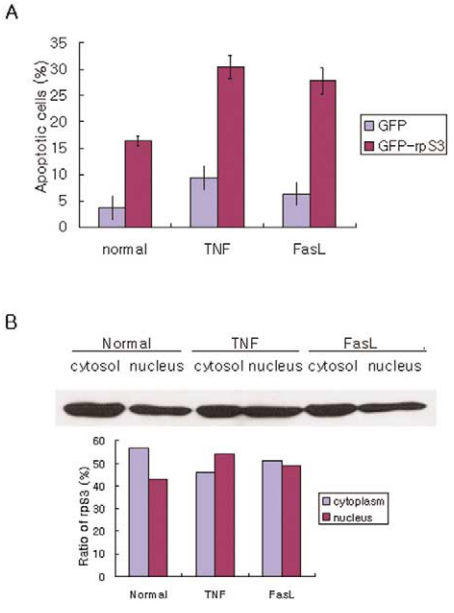

Fig. 4. RpS3 and its translocation enhance cytokine-induced apoptosis. A: RpS3 sensitizes cytokine-induced apoptosis. GFP and GFP-rpS3 were transfected to MPC-11 cells. After 24 h, cells were treated with actinomycin D (100 ng/ml). After 2 h, cells were exposed to Fas antibodies (100 ng/ml) or TNFα (30 ng/ml) for 6 h and harvested for FACS analysis. The data shown are the average of three independent experiments. B: Jurkat cells were exposed to Fas antibodies (100 ng/ml) or TNFα (30 ng/ml) for 2 h, harvested and fractionated into cytosol and nucleus. Fractions were examined by immunoblotting (IB) with anti-rpS3 antibodies. The bottom panel indicates a quantitation for the intensity of bands in the upper panel.

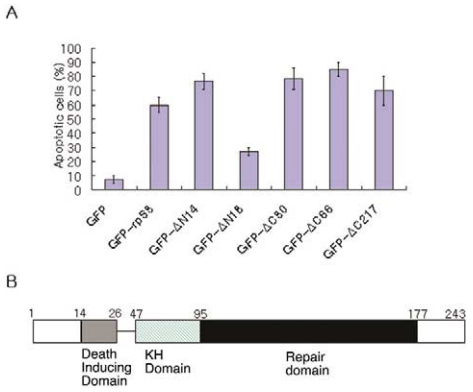

Fig. 5. RpS3 has independent domains for DNA repair and apoptosis. A: MPC-11 cells were transfected with expression vectors for GFP-fused rpS3 mutant proteins encoding N-terminal or C-terminal deletions (ΔN14, 15–243 aa; ΔN18, 19–243 aa; ΔC30, 1–213 aa; ΔC66, 1–177 aa; ΔC217, 1–26 aa). Apoptotic cells containing GFP-fused rpS3 mutants were counted by fluorescence microscopy. B: Schematic representation of human rpS3. The gray area represents the N-terminal apoptotic domain. The black box indicates the C-terminal amino acids which are sufficient for the repair endonuclease activity of rpS3. There are domains, represented by blank boxes, in both termini that appear to regulate apoptotic activities in a negative manner. KH domain is a highly conserved motif found in RNA binding proteins.

4. Discussion

RpS3, a very well-conserved protein throughout eukaryotes, with 78% amino acid similarity between *S. cerevisiae* and humans, is an essential gene in yeast, as expected [29]. In humans, it is definitely involved in DNA repair [12], implying its role in the maintenance of the integrity of the human genome as well. As a ribosomal protein, it is also related to translation, which seems to be a major function.

When cells are exposed to genotoxic stress, DNA damage processing is initiated and repair or apoptosis of the cells is determined. DNA damage-induced apoptosis seems to protect organisms from cancer generation. Transcriptional activation of p53 has been demonstrated to play a key role in the early responses induced by DNA damage [30,31]. A DNA repair enzyme might increase not only the rate of repair at the damaged site but also the rate of apoptosis under severe damage by acting as a signal mediator from repair to cell death [32]. Our study shows that ectopic expression of rpS3 protein induces programmed cell death through the activation of caspase-8 and caspase-3, indicating that rpS3 itself is a major determinant of cell fate.

NM23-H1 was identified as a granzyme A-activated DNase in a caspase-independent pathway during cytotoxic T lymphocyte-mediated apoptosis [33]. Granzyme A released from cytotoxic T lymphocytes promotes the cell death of target cells by activation of NM23-H1 and inducing single-strand DNA nicking. However, no other endonucleases responsible for DNA nicking in caspase-dependent apoptosis pathways have been identified in lymphocytes. Moreover, rpS3 mutants with N- or C-terminal deletions increased the apoptosis-inducing ability, suggesting that rpS3 might be activated by post-translational modification such as cleavage by serine proteases. It is also possible that other types of modification induce rpS3 to be involved in apoptosis.

Apoptosis, regulated by cytokines such as Fas ligand or

TNFα and caused by receptor-initiated signal transduction, plays a critical role in lymphocyte development and homeostasis. Moreover, it is well established that lymphocytes gain antigen specificity by creating a unique antigen receptor through the recombination of V, D, and J gene segments [34]. This random somatic recombination makes lymphocytes confront with genomic instability. For this reason, ATM-deficient mice develop thymic lymphomas with T cell receptor translocations at a high frequency [35]. Therefore, the proapoptotic ability of rpS3 might be significantly sensitized in lymphocytes.

This study demonstrates that rpS3 and its concurrent translocation towards the nucleus are an important event in apoptosis. Therefore, rpS3 is likely to perform its dual function as a repair enzyme and a signaling mediator of apoptosis.

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