Ribosomal Protein S3, a New Substrate of Akt, Serves as a Signal Mediator between Neuronal Apoptosis and DNA Repair

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RPS3, a conserved, eukaryotic ribosomal protein of the 40 S subunit, is required for ribosome biogenesis. Because ribosomal proteins are abundant and ubiquitous, they may have additional extraribosomal functions. Here, we show that human RPS3 is a physiological target of Akt kinase and a novel mediator of neuronal apoptosis. Nerve growth factor (NGF) stimulation resulted in phosphorylation of threonine 70 of RPS3 by Akt, and this phosphorylation was required for Akt binding to RPS3. RPS3 induced neuronal apoptosis, up-regulating proapoptotic proteins Dp5/Hrk and Bim by binding to E2F1 and acting synergistically with it. Akt-dependent phosphorylation of RPS3 inhibited its proapoptotic function and perturbed its interaction with E2F1. These events coincided with nuclear translocation and accumulation of RPS3, where it functions as an endonuclease. Nuclear accumulation of RPS3 results in an increase in DNA repair activity to some extent, thereby sustaining neuronal survival. Abolishment of Akt-mediated RPS3 phosphorylation through mutagenesis accelerated apoptotic cell death and severely compromised nuclear translocation of RPS3. Thus, our findings define an extraribosomal role of RPS3 as a molecular switch that accommodates apoptotic induction to DNA repair through Akt-mediated phosphorylation.

Nerve growth factor (NGF) deprivation and DNA damage can activate the intrinsic apoptotic pathway, causing cytochrome c release and caspase-dependent cell death in many cell types, including sympathetic neurons (1–5). NGF regulates neuronal apoptosis through a variety of cellular signaling mechanisms, especially the phosphoinositide 3-kinase (PI3K)/Akt pathway (6). Akt signaling promotes cell survival by phosphorylating and controlling downstream effectors in both the cytoplasm and the nucleus. For instance, Akt phosphorylates the proapoptotic Bcl-2 family member BAD (7) that belongs to the cytoplasmic apoptotic apparatus. In addition, Akt inhibits chromatin condensation during apoptosis by phosphorylating ACINUS, a nuclear factor required for apoptotic chromatin condensation (8). PI3K and Akt are predominantly located in the cytoplasm, but they are also found in the nucleus or translocate there upon stimulation by growth factors (9–11) or DNA damage (9–12).

Ribosomal protein S3 (RPS3) is a component of the 40 S ribosomal subunit and is involved in its maturation (13). A growing body of evidence suggests that ribosomal proteins are capable of extraribosomal functions. For example, RPS3, also known as UV endonuclease III, appears to possess a general base damage endonuclease that participates in the cleavage of DNA lesions caused by UV irradiation. In addition, both RPS3 and ribosomal protein P0 have an apurinic/apyrimidinic (AP)2 endonuclease activity functioning in DNA repair at the 3′ side of AP sites after DNA damage (14–16). In addition, ribosomal proteins may have apoptotic functions as follows: RPS3-a is involved in the apoptotic process in NIH3T3 cells (17), and knockdown of rpS3 leads to significant cell survival after hydrogen peroxide treatment (18). The exact physiological roles of RPS3 remain unclear at present.

Here, we show that Akt bound directly to RPS3 and phosphorylated it on residue threonine 70 (Thr-70). Notably, overexpression of RPS3 induced neuronal apoptosis by cooperating with E2F1 and causing up-regulation of proapoptotic BH3-only proteins, Bim and death protein 5/harakiri (Dp5/Hrk). Akt-dependent phosphorylation of RPS3 Thr-70 inhibited proapoptotic protein induction and led to nuclear accumulation of RPS3, thereby promoting cell survival through enhancing its endonuclease activity. These findings pointed to RPS3 as a key substrate for Akt and demonstrated a novel mechanism by which neuronal cells coordinate DNA repair and apoptosis.

This work was supported by Grant NI-1:22-2009-00-010-00 from the Brain Research Center of the 21st Century Frontier Research Program funded by the Ministry of Education, Science and Technology, Republic of Korea (to J.-Y.A.).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

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2 The abbreviations used are: AP, apurinic/apyrimidinic; BDNF, brain-derived neurotrophic factor; pRb, retinoblastoma protein; PARP, poly(ADP-ribose) polymerase.
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EXPERIMENTAL PROCEDURES

Cell Cultures—PC12 cells were maintained in medium A (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 5% horse serum, and 100 units of penicillin/streptomycin) at 37 °C under a 5% CO₂ atmosphere. Myc-RPS3 stably transfected PC12 cells (Tet-Off cell line) were cultured in medium B (100 μg/ml hygromycin B, 100 μg/ml G418, 2 μg/ml tetracycline in medium A). The transfected genes were induced by culturing in medium C (medium B from which the 2 μg/ml tetracycline has been removed) for 24 h. For primary culture, the hippocampi were dissected from brains of postnatal day 0 Sprague-Dawley rats and digested with 0.25% trypsin. The cells were cultured in neurobasal medium supplemented with B27, 1 mM l-glutamine, and penicillin/streptomycin. Prior to growth factor exposure, the cells were cultured in neurobasal medium supplemented with 10% fetal bovine serum, 5% horse serum, and 100 units of penicillin/streptomycin. Prior to growth factor exposure (100 ng/ml of NGF or BDNF), cells were maintained for 4–6 h under serum starvation conditions.

Antibodies, DNA, and siRNA—Anti-p-Akt, anti-Akt, anti-Bcl2, anti-Bax, anti-Bad, anti-cytochrome c, anti-PARP, anti-Lamin A, anti-caspase-9, anti-GST, anti-HA, anti-p-ERK1/2, and wortmannin were obtained from Cell Signaling (Danvers, MA). Anti-caspase-3 and active Akt proteins were obtained from Upstate (Temecula, CA). Myc-antibulin, anti-Myc, anti-GFP, and PD184352 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488 goat anti-mouse secondary antibody was obtained from Molecular Probes (Eugene, OR). PD98059 and LY294002 were obtained from Sigma.

DNA Fragmentation Assay—Oligonucleosomal fragmentation of genomic DNA was determined as described below. Briefly, 3 × 10⁶ cells transfected with rpS3 or mock vector were serum-starved for 4 h and NGF-stimulated (100 ng/ml, 45 min). After incubation, the cells were lysed on ice for 60 min in 500 μl of lysis buffer (0.02% SDS, 1% Nonidet P-40, 0.2 mg/ml proteinase K in PBS). Genomic DNA was extracted by the phenol/chloroform purification method. The pellet was dissolved in 50 μl of TE buffer (10 mg/ml RNase) for 1 h at 37 °C. 10 μg of genomic DNA was loaded on a 2% agarose gel and visualized under UV light.

Co-immunoprecipitation, in Vitro Binding, and Akt Kinase Assays—The experimental procedures for co-immunoprecipitation, in vitro binding, and Akt kinase assays have been described previously (18, 19).

In Vitro Kinase Assay—1 μg of purified protein was incubated with recombinant active Akt (Upstate) and 10 μCi of [γ-³²P]ATP (PerkinElmer Life Sciences) in 50 μl of kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na₃VO₄, 10 mM MgCl₂, and 0.2 mM ATP). Reactions were incubated at 30 °C for 30 min. Reactions were terminated by addition of Laemmli SDS sample buffer and boiling on 95 °C for 5 min. Proteins were separated on NuPAGE 4–12% gradient gels (Invitrogen), and phosphorylation of proteins was visualized by autoradiography.

Validation of Caspase Activity—Myc-RPS3-WT or mock vector were co-transfected with or without RPS3 siRNA, and transfected cells were plated onto 96-well plates (3.5 × 10⁴ cells/well). After 48 h, caspase activity was monitored by the Caspase-Glo 3/7 assay system and the Caspase-Glo 9 assay system (Promega) according to the manufacturer’s protocols.

Subcellular Fractionation—Cells (1 × 10⁶ to 5 × 10⁶ cells) were washed once with ice-cold PBS and once with lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM PMSF, protease inhibitor mixture). Cells were lysed in 200 μl of lysis buffer containing 0.1% Nonidet P-40 for 10 min on ice. The lysates were centrifuged for 2 min at 5,000 × g at 4 °C, and the supernatant was used for immunoblotting.
A nuclear pellet was washed with lysis buffer without Nonidet P-40. Nuclei were resuspended in 40 ml of nuclear protein extraction buffer (10 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA) for 10 min at 4 °C. The nuclear fraction was cleared by centrifuging at 14,000 rpm at 4 °C for 10 min. Protein concentration was measured by Bradford assay.

RT-PCR—The total RNA of Akt-inducible stable cells transfected with Myc-rpS3 was isolated with TRIzol Reagent (Invitrogen) and treated with DNase I (Ambion, Inc., Austin, TX) to remove the DNA contamination. cDNA was constructed by using Transcriptor High Fidelity cDNA synthesis kit (Roche Applied Science). PCR was performed using the following primers: Bim (forward, 5'-TGTGCGGTTGCGGCGACG-3', and reverse, 5'-TGCCGAGCAGCCAGGCCG-3') and Dp5/Hrk (forward, 5'-AGCTGAGAACCTCAGCTGTGAG-3', and reverse, 5'-AATGGTGAACCCGCAGATCTGT-3').

RESULTS

RPS3 Is a Physiological Substrate of Akt—To find a target of active Akt, we carried out immunoprecipitation assays with cellular extracts from PC12 cells constitutively expressing active Akt or mock vector as a control. Immunoprecipitated proteins were eluted and resolved by SDS-PAGE. Prominent protein bands were subjected to proteomic analysis, and RPS3 was identified as a putative binding partner of Akt. Analysis of the amino acid sequence of RPS3 revealed a single Akt consensus sequence phosphorylation site surrounding threonine residue 70 (RIRELT) in the amino terminus (Fig. 1A). We prepared a variety of recombinant GST-tagged fragments of RPS3 that included the putative phosphorylation domain, and we examined them as Akt phosphorylation targets. In vitro kinase assays in the presence of [γ-³²P]ATP revealed that only full-length RPS3 and a fragment containing amino acid residues 50–243 were significantly phosphorylated by Akt as compared with a known positive control substrate, glycogen synthesis kinase (GSK3) fusion protein (Fig. 1B).

To identify the specific RPS3 residue that is phosphorylated by Akt, we prepared several mutants His-tagged RPS3 constructs, with Thr-70 changed to alanine, aspartate, glutamate, and arginine. We also produced rabbit polyclonal antibody specific for RPS3 phosphorylated on Thr-70. All the mutations abolished RPS3 phosphorylation, whereas purified WT-RPS3 proteins tagged with histidine (His-RPS3) (supplemental Fig. S1A) were highly phosphorylated in the presence of [γ-³²P]ATP (Fig. 1C). This result strongly suggested that Thr-70 was a specific Akt phosphorylation site within RPS3. Moreover, NGF treatment triggered high levels of immunoreactivity against phospho-Akt-substrate antibody with both the wild type and the T70D mutant RPS3 proteins fused to Myc (Myc-rpS3) but

Luciferase Assay—PC12 cells were transfected with 200 ng of luciferase construct, 50 ng of β-galactosidase plasmid, various concentrations of expression vectors for E2F1, and RPS3 constructs using TransIT-LT1 (Mirus, Madison WI), according to the manufacturer’s instructions. Cells were harvested 24 h after transfection and processed with the Promega luciferase assay system. Data were normalized to β-galactosidase activity.

FIGURE 1. RPS3 is a physiological substrate of Akt. A, diagram of RPS3. RPS3 possesses a consensus Akt phosphorylation motif (RIRELT) as indicated (>). Human RPS3 sequence and functional domains are depicted. B, schematic diagram of GST-RPS3 truncation mutants (upper panel) and in vitro kinase assays (bottom panel). 1 μg of purified GST-RPS3 protein was incubated with active Akt in the presence of [γ-³²P]ATP. GSK3 fusion and GST proteins were used as positive and negative controls, respectively. C, in vitro kinase assays with RPS3 mutants. Purity of proteins was provided in supplemental Fig. S1A. D, PC12 cell lysates, transfected as shown, were immunoprecipitated (IP) and immunoblotted as indicated. E, RPS3 phosphorylation was validated by immunoblotting and immunoprecipitating with anti-RPS3. F, PC12 cells were pretreated with PI3K inhibitor (LY294002, 20 μM) or MEK inhibitor (PD184352, low, 1 μM or high, 10 μM) for 30 min, followed by NGF stimulation (100 ng/ml, 45 min). Proteins were verified by immunoblotting.
no detectable phosphorylation of the T70A Myc-RPS3 mutant. Akt was activated by NGF stimulation in all cases (Fig. 1D), suggesting that the Thr-70 residue is phosphorylated in vivo by Akt.

To investigate whether Akt can specifically phosphorylate RPS3 in intact PC12 cells, we cultured cells with or without NGF and analyzed endogenous RPS3 using specific anti-RPS3 and anti-phospho-RPS3 Thr-70 antibodies. NGF treatment elicited robust and specific phosphorylation of Thr-70 in intact cells (Fig. 1E, top panel). In addition, an antibody against phosphorylated Akt substrate specifically interacts precipitated RPS3 protein (Fig. 1E, 3rd panel). To further explore NGF-mediated Akt phosphorylation of RPS3, PC12 cells were treated with either a PI3K inhibitor (LY294002) or a MEK1 inhibitor (PD184352), followed by NGF stimulation. Endogenous RPS3 was highly phosphorylated upon NGF stimulation. Phosphorylation was strongly inhibited by the PI3K inhibitor but not by the MEK1 inhibitor, suggesting that the PI3K/Akt pathway, and not the MAPK pathway, is responsible for Thr-70 phosphorylation of RPS3 (Fig. 1F). A quantification analysis was provided in supplemental Fig S1B. Taken together, our data support the conclusion that RPS3 is a physiological substrate of Akt in mammalian cells.

Akt Physically Interacts with RPS3—In vitro mapping experiments using a variety of purified GST–RPS3 proteins (Fig. 1B) and lysates prepared from cells expressing hemagglutinin-tagged Akt (HA-Akt) demonstrated that, besides RPS3-WT, the only fragment that interacted robustly with Akt was RPS3 residues 50–243 (Fig. 2A). This result was in agreement with our in vitro phosphorylation findings (Fig. 1B) and suggested that RPS3 phosphorylation was critical for Akt binding and/or for the maintenance of RPS3 conformation. To test whether Thr-70 phosphorylation caused RPS3 to bind to Akt, we introduced a constitutively active, phosphorylation-mimetic mutant
HA-Akt (T308DS473D) into PC12 cells that were expressing either RPS3-WT or RPS3-T70A. Active Akt clearly associated with RPS3-WT and not with the T70A mutant, suggesting that Akt facilitated its association with RPS3-WT by phosphorylating it (Fig. 2B, top). We noted that the Thr-70 phosphorylation status of precipitated RPS3 was highly correlated with Akt binding (Fig. 2B, bottom). NGF treatment of PC12 cells led to a substantial increase in binding of endogenous RPS3 to Akt, further suggesting that Akt phosphorylation of RPS3 promoted its interaction with RPS3 (Fig. 2C).

To determine the function of RPS3 Thr-70 phosphorylation, we established PC12 cell lines stably transfected with tetracycline-inducible Myc-RPS3-WT, T70A or T70D constructs (supplemental Fig. S2A). In the absence of NGF stimulation, endogenous Akt associated with the RPS3-T70D mutant but showed negligible binding to RPS3-WT or to the T70A mutant (Fig. 2D, top left). In contrast, NGF treatment resulted in a robust interaction between RPS3-WT and Akt, and the binding affinity of this interaction appeared similar to that of Akt to the T70D mutant (Fig. 2D, top right). Interestingly, Akt binding levels in the T70A mutant cell line were independent of NGF stimulation. We concluded that NGF stimulated the RPS3-Akt interaction, presumably by activating Akt, which subsequently leads to the phosphorylation of RPS3 Thr-70 (Fig. 2D, left and right, 2nd panel). In addition, NGF-induced Akt-RPS3 complex formation was disrupted by the PI3K inhibitor (LY294002) but was not affected by the MEK inhibitor (PD98059), again arguing that Akt activation was required for the Akt-RPS3 interaction (supplemental Fig. S2B). Mapping experiments demonstrated that RPS3 bound full-length Akt or fragments containing the catalytic domain but bound only very weakly to fragments containing either the regulatory domain or the pleckstrin homology domain (Fig. 2E). The critical role of the catalytic domain of Akt in RPS3 binding supported the idea that Akt kinase activity is crucial for its interaction with RPS3.

**RPS3 Induced Neuronal Apoptosis That Was Inhibited by NGF**—During the preparation of both stably and transiently transfected cell lines, we noticed that apoptotic cell death was occurring in RPS3-overexpressing cell lines, especially in the cells expressing T70A mutant RPS3. These observations raised the question of whether RPS3 played a role in neuronal apoptosis. We found that overexpression of GFP-RPS3 caused pronounced DNA fragmentation that was compromised by NGF treatment, indicating that overexpression of RPS3 could indeed lead to neuronal cell death (Fig. 3A). Cleavage of caspase-3 and its well known downstream target proteins, PARP and Lamin A/C, were coupled to the DNA fragmentation activity. Akt was also activated upon NGF treatment, implying that RPS3 may possess a proapoptotic function and that this activity could be compromised by NGF-mediated Akt activation (Fig. 3B). Compared with cells expressing control vector, more than 50% of the nuclei in GFP-RPS3-expressing PC12 cells displayed condensed chromatin, and NGF treatment decreased chromatin condensation by less than 20% (Fig. 3C). Primary hippocampus neurons, but not control cells, exhibited more than 40% of chromatin condensation in the presence of overexpressed RPS3 (Fig. 3D). We concluded that RPS3 was crucial for induction of neuronal cell death.

To examine whether RPS3 expression levels affected apoptosis, we depleted RPS3 expression by short interfering (si) RNA. Depletion of endogenous RPS3 resulted in modest apoptosis (15%) as compared with scrambled RNA-transfected cells, suggesting that abrogation of RPS3 expression could also trigger apoptosis and was marginally compromised by NGF (Fig. 3E), whereas overexpression of RPS3 resulted in higher levels of cell death (more than 50%). Stepwise removal of RPS3 from GFP-RPS3-overexpressing cells clearly reduced apoptosis, and NGF exposure substantially enhanced neuronal cell survival (Fig. 3E). As reported previously (20), cell death resulting from the elimination of RPS3 may be due to a perturbation of protein translation. Hence, these findings strongly supported the idea that enrichment of RPS3 protein was a potent trigger of apoptotic cell death, which in turn could be repressed by NGF. On the other hand, knockdown of RPS3 led to a decrease of the resistance to neuronal apoptosis that was induced by etoposide (Fig. 3F, left), indicating that RPS3 is critical for neuronal cell survival. However, compared with overexpression of RPS3, knockdown of RPS3 resulted in a substantial decrease in the levels of proapoptotic proteins, such as Bim and DP5, as well as cleavage of caspase-3 (Fig. 3F, right), supporting our finding that high levels of RPS3 expression resulted in substantial apoptosis, although depletion of RPS3 might also result in apoptosis. Collectively, our data may explain the impression that dual function of RPS3 as an individual ribosomal protein and as an apoptotic inducer of neuronal cells.

**RPS3 Induced Cytochrome c Release and Apoptosome-dependent Death**—Overexpression of RPS3 not only stimulated DNA fragmentation but also increased the degradation of PARP and Lamin A/C (Fig. 3, A and B), and these effects were abolished by the removal of overexpressed RPS3 (Fig. 3E). These findings implicated RPS3 in multiple levels of apoptotic induction, including activation of upstream apoptotic machinery like caspase-3. To assess this possibility, we monitored the effect of RPS3 depletion on activation of the caspase cascade. Silencing of endogenous rpS3 slightly increased either caspase-9 or caspase-3 activity as compared with the control and regardless of NGF stimulation. Notably, cells expressing Myc-RPS3 displayed an ~2-fold higher level of activated caspase-9 and a 4-fold higher level of activated caspase-3 compared with control cells in the absence of NGF, and NGF treatment decreased RPS3-mediated caspase-9 and caspase-3 activities to basal levels (Fig. 4, A and B). Correspondingly, ablation of RPS3 in RPS3-overexpressing cells caused a reversion of caspase-9 and -3 activities, indicating that RPS3 may act upstream of caspase-3, most likely in the mitochondrial apoptotic pathway.

Because neurons undergoing apoptosis mediated by the apoptosome pathway require the release of cytochrome c and the execution of the caspase cascades, we tested whether apoptotic death was triggered by RPS3-induced apoptosome activation. Overexpression of RPS3 resulted in not only activation of caspase-3 (Fig. 4C, 1st and 2nd panels from the top) but also cytochrome c release and caspase-9 activation in the absence of NGF, indicating engagement of the apoptosome pathway (Fig. 4C, 3rd panel). Importantly, Bim and Dp5/Hrk but not Bcl-2 or Bax levels were up-regulated in RPS3-expressing cells (Fig. 4C, 4th to 7th panels). NGF exposure dramatically diminished both...
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*Bim* and *Dp5* protein levels (Fig. 4C, 6th and 7th panels from the top). By using luciferase reporter plasmids containing *Bim* or *Dp5* promoters (pGL4/Bim or pGL4/Dp5), we demonstrated that transcription levels from both these promoters increased up to 3-fold relative to the control vector (pGL) with increasing Myc-RPS3 (Fig. 4D). The protein expression was provided in supplemental Fig. S3A. RPS3 may therefore regulate not only protein but also transcription levels of *Bim* and *Dp5*.

Although the precise mechanism whereby *Bim* and *Dp5* mediate neuronal apoptosis is uncertain, both *Bim* and *Dp5* are...
up-regulated after NGF withdrawal in sympathetic neurons (21–23). A recent study suggested that E2F1 binds to the promoters of both *Bim* and *Dps5*, enhancing their expression (24). To understand the molecular basis of RPS3-mediated up-regulation of proapoptotic proteins, we tested whether overexpression of RPS3 affected E2F1. RPS3-WT interacted with endogenous E2F1 in the absence of NGF, but no binding was observed in the presence of NGF (Fig. 4E and supplemental Fig. S3B). Moreover, co-transfection of RPS3 and E2F1 significantly increased transcription of both *Bim* and *Dps5* compared with expression levels using E2F1 or RPS3 on their own (Fig. 4F). Thus, our data suggested that RPS3 acted synergistically with E2F1 to produce an increase in BH3-only protein levels in the absence of NGF and dissociated from E2F1 in response to NGF stimulation.

To assess the function of the RPS3-E2F1 interaction in neuronal apoptosis, we used siRNA to reduce the expression of E2F1 in PC12 cells that had been induced to undergo apoptosis by overexpression of RPS3. As expected, overexpression of RPS3 and control scrambled short RNAs still induced apoptosis, leading to cleavage of both caspase-3 and PARP (Fig. 4G, 2nd lanes). However, depletion of E2F1 significantly inhibited RPS3-induced neuronal cell death (Fig. 4G, 3rd lanes). We used DAPI staining to quantitate the ratio of apoptotic to nonapoptotic cells (supplemental Fig. S3C). Together, these results showed that binding of E2F1 to RPS3 contributed to RPS3-induced apoptosis in neuronal cells. Because we failed to detect any changes in either JNK or c-Jun activation (data not shown), our results suggested that RPS3 may function proapoptotically by interacting with E2F1 and up-regulating BH3-only protein levels, whereas NGF-driven Akt signaling antagonized this effect, independent of JNK signaling.

Akt Phosphorylation Suppressed RPS3-induced Apoptosis—Because Thr-70 phosphorylation of RPS3 by Akt appeared to regulate its binding to E2F1 as well as the induction of proapoptotic proteins, we next asked whether Akt phosphorylation influenced RPS3-mediated apoptosis in neuronal cells. Consistent with the observation shown in Fig. 3A, DNA fragmentation was higher in cells expressing RPS3 compared with control PC12 cells, whereas NGF exposure decreased RPS3-mediated DNA fragmentation. Importantly, RPS3 T70D phosphorylation-mimetic mutant cells exhibited reduced DNA fragmentation, regardless of NGF treatment. In contrast, the RPS3 T70A cells showed significant DNA fragmentation even in the presence of NGF, demonstrating that phosphorylation of RPS3 by Akt is required to prevent RPS3-mediated apoptosis (Fig. 5A, left). A quantitative analysis of apoptosis by DAPI staining correlated well with DNA fragmentation activity (Fig. 5A, right). More than 60% of the cells underwent apoptosis in both RPS3 WT and RPS3 T70A mutant cell lines. NGF stimulation reduced this number to 38% in WT cells, and than 50% of the RPS3 T70A mutant-transfected cells showed chromatin condensation, again supporting the conclusion that Akt phosphorylation is critical for suppressing RPS3-induced apoptosis. We observed similar results in PC12 cells that were transiently transfected with GFP-RPS3-WT or with T70A or T70D mutants (data not shown). The same results were obtained by FACS analysis with annexin V staining (Fig. 5B). We then expanded our study to primary hippocampus neurons. Neurons infected with adenovirus expressing HA-RPS3-T70D did not display altered nuclei, regardless of the presence of BDNF. In contrast, HA-RPS3-WT- or HA-RPS3-T70A-infected neurons underwent apoptotic death more frequently in the absence of BDNF than when BDNF was present, underscoring the critical role of Thr-70 phosphorylation in suppression of RPS3-induced neuronal apoptosis (Fig. 5C and supplemental Fig. S4; a quantitative analysis is summarized in Fig. 5D).

Akt Phosphorylation Governed Nuclear Accumulation of RPS3—Next, we investigated whether Akt phosphorylation affected translocation of RPS3 from its primary location in the cytoplasm to the nucleus. We found that endogenous RPS3 translocated from the cytoplasm to the nucleus, exhibiting a dynamic similar to that of Akt upon NGF stimulation and underscoring its novel, extraribosomal function in NGF signaling (Fig. 6A). Our subcellular fractionation assay after NGF treatment demonstrated that phospho-RPS3 accumulated in the nucleus in a time-dependent manner and coincident with a decrease in cytoplasmic phospho-RPS3 levels (Fig. 6B, 1st panel). This was similar to the translocation pattern of active Akt after NGF stimulation (Fig. 6B, 3rd panel) and consistent with our previous observation that Akt translocates to the nucleus after a 20–30-min treatment with NGF (11). To verify the nuclear localization of phospho-RPS3, we performed immunocytochemistry on PC12 cells that were co-transfected with various Myc-RPS3 constructs and a red fluorescent protein-fused Akt protein (RFP-Akt). RFP-Akt was detected mainly in the cytoplasm but was also present in the nucleus upon NGF treatment. We observed translocation of RPS3-WT to the nucleus, where it co-localized with active Akt after NGF exposure. The RPS3-T70D mutant predominantly localized to the nucleus, whereas the RPS3-T70A mutant mainly occurred

**FIGURE 3. RPS3 induced neuronal apoptosis and is regulated by NGF.** A. PC12 cells, transfected as indicated, were treated with NGF (100 ng/ml, 45 min), and 10 μg of genomic DNA was separated on a 2% agarose gel. B, PC12 cells, transfected as indicated, were immunoblotted as shown. C and D, PC12 cells (C) or primary hippocampal neurons (D), transfected as indicated, with or without NGF stimulation (100 ng/ml, 45 min) or starved in neurobasal medium without B27 for 1 h. Scale bar = 20 μm (C) or 10 μm (D). Fixed cells were stained with Hoechst (C, left, 1st panel) or DAPI (D, left, middle panel) (blue). Arrows indicate apoptotic cells with condensed chromatin morphologies. A quantitative apoptotic rate analysis of condensed chromatin versus total transfected cells is shown (right). E, RPS3 siRNA (si-rp32) or scrambled siRNA (scriRNA) was simultaneously transfected with mock vector or Myc-RPS3 into PC12 cells. Genes were expressed for 48 h, and cells were serum-starved for 4 h, followed by NGF stimulation (100 ng/ml, 45 min). PC12 cells were stained with DAPI (blue). The left panel shows a representative picture. Scale bar, 20 μm. A quantitative analysis of the apoptotic rate is shown on the right. This and in all other figures, apoptotic rate was determined from 500 cells in different fields and calculated as the mean ± S.D. of three independent experiments. Morphological changes in the nuclear chromat of cells undergoing apoptosis were detected by DAPI or Hoechst staining. Only the nuclei possessing multiple condensed and aggregated chromatin masses were counted. F, minor activity of knockdown of RPS3. PC12 cells were transfected as indicated. Genes were expressed for 48 h, and etoposide (25 μm) was treated for 15 h. Effect of knockdown of RPS3 was analyzed by immunoblotting and measurement of caspase-3 activity (left panel). Induction rates of proapoptotic proteins (Bim, Dps5/Hrk, active caspase-3) with loading controls were analyzed by immunoblotting (right panel). All data were statistically analyzed by Student’s t test against control, and arrows indicate condensed chromatin morphologies, *, p < 0.05; **, p < 0.005.
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![Graphs and images](image)

**FIGURE 4.** RPS3 induced cytochrome c release and apoptosome-dependent death. A and B, caspase-9 or -3 activity. RPS3 siRNA (si-rpS3) or scrambled siRNA was transfected with mock vector or Myc-RPS3 into PC12 cells. Caspase-9 or caspase-3 activity was analyzed using the caspase Glo 9 or caspase Glo 3/7 assay kit (Promega), respectively. C, PC12 cells were transfected with Myc-RPS3 WT or mock control constructs with or without NGF stimulation. Immunoblots are shown for the indicated antibodies. D, PC12 cells were co-transfected with control (Ctrl) pcDNA vector or Myc-RPS3, and with a luciferase reporter plasmid under control of a Bim− or Dp5 promoter, or with a control vector (pGL). Transcriptional activities of Bim and Dp5 were measured as the level of RPS3 gradually increased. E, Myc-RPS3 expressing cells were immunoprecipitated (IP) and immunoblotted as indicated with or without NGF stimulation. Equal amounts of protein were used as provided in supplemental Fig. S3A. F, relative RPS3 (+, +++; ++, ++; or +++, +++) were determined the endonuclease activity of RPS3 in response to DNA damage. The regulation of the nuclear accumulation of RPS3 by Akt-mediated phosphorylation of Thr-70 supports the hypothesis that Akt may function on DNA damage by phosphorylating RPS3, thereby activating the endonuclease activity of RPS3 in response to DNA damage. To test this hypothesis, we determined the endonuclease activity of purified His-RPS3 WT, T70A, or T70D mutants (supplemental Fig. S5A) against AP-damaged DNA. We included Nth, known as endonuclease III, as a positive control to validate our endonuclease assay. With intact DNA, all RPS3s barely caused nicks in normal plasmid DNA, probably the result of a background level of nonspecific enzymatic activity. However, RPS3 WT and T70A introduced nicks at AP sites to a similar extent, whereas RPS3 T70D produced a larger amount of nicks than did the WT or T70A (Fig. 7A, upper panel). RPS3 WT protein has around a 10-fold stronger nicking activity at AP sites than does the control, and the T70A mutant has slightly less nicking activity than does RPS3 WT. The T70D mutant had a 13-fold higher endonuclease activity than the control against damaged DNA, suggesting that Akt phosphorylation of Thr-70 on RPS3 may enhance its endonuclease activity.

Akt Phosphorylation Suppresses Apoptotic Activity of RPS3 by Enhancing Its Endonuclease Activity—Akt is activated by various DNA damage conditions and has effects on the cell cycle and apoptotic parameters, promoting cell survival (12, 27). Because Akt provides a prosurvival signal for the cell in response to DNA damage, it is possible that Akt may regulate the endonuclease activity of RPS3 for making a critical decision whether the cell enters a repair cycle or a cell death program upon the extent of DNA damage. The regulation of the nuclear accumulation of RPS3 by Akt-mediated phosphorylation of Thr-70 supports the hypothesis that Akt may function on DNA damage by phosphorylating RPS3, thereby activating the endonuclease activity of RPS3 in response to DNA damage. To test this hypothesis, we determined the endonuclease activity of purified His-RPS3 WT, T70A, or T70D mutants (supplemental Fig. S5A) against AP-damaged DNA. We included Nth, known as endonuclease III, as a positive control to validate our endonuclease assay. With intact DNA, all RPS3s barely caused nicks in normal plasmid DNA, probably the result of a background level of nonspecific enzymatic activity. However, RPS3 WT and T70A introduced nicks at AP sites to a similar extent, whereas RPS3 T70D produced a larger amount of nicks than did the WT or T70A (Fig. 7A, upper panel). RPS3 WT protein has around a 10-fold stronger nicking activity at AP sites than does the control, and the T70A mutant has slightly less nicking activity than does RPS3 WT. The T70D mutant had a 13-fold higher endonuclease activity than the control against damaged DNA, suggesting that Akt phosphorylation of Thr-70 on RPS3 may enhance its endonuclease activity at AP sites (Fig. 7A, bottom). To confirm that Akt phosphorylation regulates the endonuclease activity of RPS3, we assessed the endonuclease activity of RPS3 against specific kinase inhibitors on the subcellular localization of RPS3. Pretreatment with the PI3K inhibitor decreased nuclear localization of both RPS3 and phospho-RPS3 after NGF stimulation (Fig. 6D), suggesting that Akt phosphorylation governs RPS3 nuclear accumulation that in turn impairs its apoptotic function thereby promoting cell survival.

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plasmid DNA cross-linked upon UV irradiation. RPS3-WT cleaved UV-damaged DNA at a higher rate than did the control. However, the T70A mutant hardly digested the plasmid DNA, showing a smeared fragment, whereas the RPS3-T70D mutant caused clear nicking of the UV-damaged DNA, implying that Akt phosphorylation enhances DNA excision activity (Fig. 7B). Notably, the endonuclease activity of phospho-RPS3 is higher than that of unphosphorylated RPS3 by performing an in vitro kinase assay using Akt immunoprecipitates in the presence or absence of ATP to ensure that RPS3 was phosphorylated. This was followed by a plasmid-nicking assay conducted in a time-dependent manner after UV irradiation (Fig. 7C). Thus, our experiment demonstrated that Akt up-regulates the endonuclease activity of RPS3 via phosphorylation and led us to believe that Akt phosphorylation of RPS3 after DNA damage is an antiapoptotic signal or a molecular switch that extends the life of a cell after DNA damage.

The recruitment of DNA repair proteins to damaged DNA could be the first event triggered by DNA damage, addressing the reason for the nuclear accumulation of RPS3 upon Akt phosphorylation (Fig. 6). As NGF treatment drives RPS3 to the nucleus via Akt phosphorylation, disturbing its proapoptotic function, we wondered whether DNA damage also regulated nuclear accumulation of RPS3, bringing the repair enzyme into the vicinity of its substrate and active Akt into the vicinity of its kinase substrate. Indeed, phosphorylated Akt was also observed in the nucleus after exposure to various genotoxic stresses (supplemental Fig. S5B), and the levels of both endogenous RPS3 and phosphorylated RPS3 increased and accumulated in the nucleus after UV damage in cultured primary neurons (supplemental Fig. S5C). Moreover, we also showed that Akt is activated in response to DNA damage to some extent and that RPS3 phosphorylation is correlated with Akt phosphorylation in a time-dependent manner upon both UV irradiation and etoposide treatment (Fig. 7D). Fig. 7E (left panel) depicts co-localization of endogenous Akt and phosphorylated RPS3 in the nucleus upon UV irradiation in primary hippocampal neurons. The quantitative ratio for nuclear localization with Z-stack was summarized in Fig. 7E (right panel). Thus, our data revealed the existence of an exclusively nuclear, active Akt after DNA damage near its substrate, RPS3, and that this co-localization may enhance the phosphorylation of RPS3, subsequently up-regulating its endonuclease activity and promoting cell survival.

DISCUSSION

Our work provides a detailed characterization of RPS3 as a physiological substrate of Akt and suggests the likely involvement of an individual ribosomal protein in regulating neuronal apoptosis. Akt physically bound to RPS3 and phosphorylated it on residue Thr-70. Overexpression of RPS3 in PC12 cells and
primary neurons markedly induced apoptotic cell death, permitting cytochrome c release and activation of the apoptosome pathway, including expression of the BH3-only proteins Bim and Dp5/Hrk. Remarkably, RPS3 associated with E2F1, thereby promoting proapoptotic gene induction. However, phosphorylation of RPS3 by Akt was critical for suppression of its proapoptotic function, mediating its dissociation from E2F1 and forcing its localization in the nucleus. Importantly, we suggest a novel mechanism for neuronal survival by active Akt, through regulation of the proapoptotic function of an individual ribosomal protein.

The cessation of ribosome biogenesis and disintegration of the translational apparatus ultimately result in cell death, and a recent study showed that RPS3 is essential for pre-18S rRNA processing (28). Indeed, we observed that modest apoptotic cleavage of PARP and chromatin condensation resulted after depletion of endogenous RPS3 (Fig. 3, E and F). However, RPS3 silencing caused much lower levels of apoptosis than overexpression of RPS3. The regulatory function of RPS3 in apoptosis makes it the newest member of a growing list of ribosomal proteins with extraribosomal functions (29). Overexpression of RPS3 specifically induced Bim and Dp5, producing an effect similar to trophic withdrawal in PC12 cells (Fig. 4, C and D). One of the prominent features of BH3-only proteins is their elevated expression levels in the presence of E2F1 (24). E2F1 transcriptional activity is repressed by direct interaction with retinoblastoma protein (pRb) (30). Interestingly, a recent study suggested that the pRb-E2F1 complex formation does not change in response to DNA damage; instead, pRb participates in transcriptional activation of proapoptotic genes (31). We also observed co-immunoprecipitation of pRb and RPS3, regardless of NGF stimulation (data not shown), whereas RPS3 physically interacted with endogenous E2F1 only in the absence of growth factor (Fig. 4, E, top). In addition, neither overexpression nor knockdown of RPS3 affected the interaction between E2F1 and Rb (supplemental Fig. S3D). We assumed that RPS3 may participate in a tricomplex with E2F1-pRB and that the association of RPS3 and E2F1 may promote the induction of BH3-only proteins in the absence of growth factor. Instead, growth factor stimulation led to phosphorylation of RPS3 that caused dissociation of both RPS3 and pRb from this complex, thereby inducing transcriptional activation of E2F1 target genes that are required for DNA synthesis. However, it is not clear whether RPS3 acts as a transcriptional activator or a coactivator. It will be interesting to determine

FIGURE 6. Akt phosphorylation governs the nuclear accumulation of RPS3. A, PC12 cells were transfected with RFP-Akt, immunostained with anti-RPS3, and visualized by anti-rabbit Alexa Fluor 488 antibody (green) under a fluorescence microscope. Nuclei were counter-stained with DAPI. Scale bar, 20 µm. B, PC12 cells were stimulated by NGF for 0, 10, 30, or 60 min, and cytoplasmic (Cyto) and nuclear fractions (Nu) were separated. Proteins were analyzed by immunoblotting with the indicated antibodies (top). A quantitative analysis of p-RPS3 was provided (bottom). C, PC12 cells, co-transfected as indicated, were immunostained as shown and visualized by anti-rabbit Alexa Fluor 488 antibody (green) under the same conditions as A. Scale bar, 20 µm (top). A quantitative analysis of nuclear translocated cells was provided (bottom). D, cells were pre-treated with LY294002 (20 µM) for 30 min, followed by NGF stimulation. The cytoplasmic (C) and nuclear (N) fractions were analyzed by immunoblotting with the indicated antibodies (top panel). A quantitative analysis was provided (bottom panel). All data were statistically analyzed by Student’s t test against control (Ctrl). *, p < 0.05; **, p < 0.005.
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how RPS3 enhances E2F1 transcriptional activity and how specific signals cause the dissociation of RPS3 from E2F1, an event that appears to be essential for the cooperative function of RPS3 and E2F1.

RPS3 was robustly phosphorylated by Akt, but the T70A mutant form of RPS3 could not be phosphorylated (Fig. 1), implicating RPS3 Thr-70 as the target of Akt phosphorylation. Because we mostly detected phosphorylated RPS3 upon NGF stimulation in the cytosolic fractions, although RPS3 is distributed in both the ribosome free-cytosolic fraction and ribosomal fraction (supplemental Fig. S6, A and B), we assumed that the cytosolic fraction of RPS3 plays the critical role in Akt-mediated signaling. Moreover, RPS3-WT strongly binds to Akt, whereas the T70A RPS3 mutant did not bind to Akt at all, suggesting that RPS3 phosphorylation is critical for its interaction with Akt (Fig. 2, B and C). Furthermore, Akt phosphorylation of RPS3 appeared necessary for inhibiting its proapoptotic function, because NGF stimulation repressed the proapoptotic activities of RPS3-WT and RPS3-T70D but not that of RPS3-T70A (Fig. 5).

RPS3 is a dynamic protein that shuttles between the cytoplasm and the nucleus and functions in both compartments. RPS3-WT was distributed throughout the cytoplasm and nucleus but translocated to the nucleus upon NGF treatment or DNA damage conditions (Fig. 6, A and B, Fig. 7D, and supplemental Fig. S5C). Interestingly, Akt phosphorylation augmented nuclear accumulation of RPS3 and ablated its proapoptotic effect (Figs. 6B and 5). Moreover, the RPS3-T70A mutant barely translocated to the nucleus, whereas the T70D phospho-mimetic mutant resided predominantly in the nucleus, even in the absence of any cellular stimuli (Fig. 6C). Our findings suggest that the function of RPS3 in neuronal apoptosis may be conferred by its distinct subcellular localization in neurons. Mounting evidence shows that Akt contributes to the prevention of apoptosis by phosphorylating proapoptotic proteins and/or changing the localization patterns of its substrates, driving them away from their functional sites. Thus, it is conceivable that Akt phosphorylation of RPS3 removes RPS3 from the cytoplasm where it normally contributes to apoptosis by enhancing the levels of the proapoptotic Bim and Dp5 proteins. Akt may also be involved in the regulation of other possible nuclear functions of RPS3. Remarkably, RPS3 translocates to the nucleus upon T cell receptor and TNF stimulation where it becomes a subunit of the nuclear NF-κB complex in Jurkat cells (26). Likewise, it will be worthwhile to determine how Akt-dependent phosphorylation mediates nuclear translocation of RPS3, specifically antagonizing its proapoptotic function in neurons.

Although it is not known yet how Akt is activated in response to various genotoxic treatments, recent studies have shown that Akt is activated by various DNA damage conditions and is known to affect both the cell cycle and apoptotic parameters to...
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promote cell survival (12, 32). Indeed, we also observed that Akt was activated to some extent upon DNA damage, and RPS3 phosphorylation was correlated with Akt phosphorylation in the nucleus (Fig. 7E and supplemental Fig. S5B). Because in dividing cells RPS3 is involved in DNA damage recognition through its endonuclease activity (18, 33), and we found that Akt phosphorylation of RPS3 prevented its proapoptotic function (10, 11). Another remaining question is whether the apoptotic activation and enhanced its endonuclease activity after DNA damage, it is conceivable that Akt promotes cell survival by regulating the DNA repair activity of RPS3 and allows some time for cells to decide whether to live or die. Clearly, a better understanding is needed of how DNA damage translates into a pro-survival signal to escape cell death.

The decision that a cell activates a repair or a death program may be affected by the extent of DNA damage. A few damaged points on the DNA could be repaired by endonucleases. However, excess excisional repair of multiple impaired nucleotides may cause irreversible damage, eventually leading to cell death. We noticed that Akt activation in response to various types of DNA damage was coupled to limited PARP cleavage. Once severe cleavage of PARP occurred, Akt phosphorylation declined (Fig. 7D and supplemental Fig. S5B). This suggests that Akt phosphorylation of RPS3 after DNA damage may be a molecular switch, whereby cellular survival is favored over additional DNA destruction mediated by cellular factors. Previous studies have shown that DNA damage can activate the intrinsic apoptotic pathway leading to cytochrome c release and caspase-dependent cell death in many cell types, including sympathetic neurons (3, 4). Our findings provide a possible explanation of how cells regulate DNA repair and cell death. Another remaining question is whether the apoptotic activation function of RPS3 contributes to the pathogenesis of neurological disorders. Because Akt selectively initiates anti-apoptotic defenses and represses programmed cell death by regulation of RPS3, manipulation of nuclear RPS3 and its phosphorylation by Akt may provide useful clues in the development of novel treatments for neurological diseases.

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