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Biochemical and Biophysical Research Communications 333 (2005) 110–115
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Erk phosphorylates threonine 42 residue of ribosomal protein S3

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Received 13 May 2005
Available online 31 May 2005

Abstract

The ribosomal protein S3 (rpS3) is involved in ribosome biogenesis as a member of ribosomal small subunit and also plays a role in the repair of damaged DNA. Extracellular signal-regulated kinase (Erk), a MAP kinase, is known to play important roles in the regulation of cell growth, differentiation, and apoptosis. In this study, the sequence analysis of rpS3 protein revealed that this protein has a putative FXFP motif which is believed to be an Erk binding site. Indeed, the motif was demonstrated as an Erk binding site by co-immunoprecipitation. In addition to this, it was revealed that Erk specifically phosphorylated Thr 42 residue of rpS3 in vitro and in vivo using the various mutants of rpS3. Taken together, rpS3 appears to be phosphorylated by activated Erk in proliferating cells, resulting in the decreased interaction between two proteins.

Keywords: rpS3; Erk; Phosphorylation; Threonine residue; FXFP motif

Mitogen-activated protein kinases (MAPKs) are signal transducers that transmit signals of extracellular stimuli from the cell surface to the nucleus. The family of MAPKs, including extracellular-regulated kinase (ERK), c-jun amino-terminal kinase (JNK), and p38, are activated by phosphorylation of their threonine and tyrosine residues at a TXY motif in the activation loop [1]. The phosphorylation of MAPKs is regulated by their specific activator MAPK kinases (MAPKKs or MEKs) [2,3] and repressor of various phosphatases [3]—tyrosine phosphatases and dual-specificity MAPK phosphatases (MKPs). Once activated, MAPKs catalyze the phosphorylation of their targets, including transcription factors and MAPK-activated protein kinases (MAPKAPKs). Recent studies demonstrate the specific targeting of MAPKs to substrates via a docking domain. Two docking sites for MAPKs have been identified on substrates. The δ domain/D box mediates the interaction with MAPKs [4,5], and the amino acid sequence FXFP mediates the interaction with Erk but not with other MAPKs [5–7]. For Erk, many different proteins have been reported to be substrates which include signaling proteins that function upstream of Erk such as MEKs and function downstream of Erk such as MAPKAPKs, transcription factors, and proteins [6].

Ribosomal protein S3, a 26.7 kDa protein, is a member of ribosomal small subunit and is also known to be involved in the initiation of translation. It was cross-linked to eukaryotic initiation factors, eIF-2 [8] and eIF-3 [9]. Interestingly, it has also been reported that this protein has an extra-ribosomal function, so-called UV endonuclease III, to repair the DNA damage by UV irradiation [10–13]. This enzyme cleaves UV irradiated DNA, and DNA containing AP (apyrimidic/apurinic) sites or thymine glycols, by cleaving the phosphodiester bond within a cyclobutane pyrimidine dimer of the UV irradiated DNA or the 3′ end of the lesion such as AP sites via a lyase mechanism [14–16].

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.05.079

RpS3 proteins have several conserved motifs: a nuclear localization signal—KKRK, a KH (hnRNP K homology)—RNA binding domain [17,18], and a S3-C domain are well conserved among the eukaryotes.

Generally, most ribosomal proteins are believed to have post-translational modifications such as phosphorylation [19], ubiquitination [20], acetylation [21], and methylation [22]. One well-known modification is phosphorylation that mainly occurs on serine and threonine residues. The phosphorylation appears to result in the control of the stability of ribosomal proteins or ribosome biogenesis. For instance, the phosphorylation of ribosomal protein S6 was derived from activated S6K by exogenous and intracellular signals, and it has recently been implicated in the translational up-regulation of mRNAs coding for the components of protein synthetic apparatus [23,24]. Upon phosphorylation of the ribosomal protein L11 is released from the ribosome complex, and then it functions as a negative regulator of HDM2 and brings about the p53-dependent cell cycle arrest [25].

In this study, we examined whether rpS3 interacts with Erk MAPK, and the interaction elicits the phosphorylation of the rpS3 protein.

Materials and methods

Plasmids and site-directed mutagenesis. A full length of human rpS3 gene was cloned in-frame with a sequence coding for EGFP (pEGFPc1, Clontech), His (pET21a, Novagen), and GST (pGEX5x-1, Amersham Biosciences). Site-directed mutagenesis was performed by overlap extension using the polymerase chain reaction (PCR). The rpS3 gene cloned in pGEX5x-1 vector was used as a template for mutagenesis. Two sets of PCRs were carried out: the first PCR fragment was synthesized using rpS3-forward primer and an antisense mutagenic primer. The second PCR fragment was synthesized using rpS3-reverse primer and a sense mutagenic primer. The two amplified products were mixed, annealed, and polymerized by Taq polymerase (Takara). Using this product as a template, PCR was performed with rpS3-forward and -reverse primers.

Protein expression and purification. Escherichia coli strain BL21(DE3)/pLysS (Novagen) cells were transformed with the pET21a and pGEX5x-1-based plasmids. The transformed cells were grown overnight in LB broth containing 50 μg/ml ampicillin at 37 °C, and then one-hundredth of the cells were grown in LB broth containing 50 μg/ml ampicillin, 0.4% dextrose at 30 °C until the cell density reached $A_{600} = 0.6$–0.8. The recombinant proteins were expressed after induction with 0.5 mM isopropyl-1-β-D-galactopyranoside (IPTG), and purified with glutathione–Sepharose 4B (Pharmacia) and Ni^{2+}–NTA beads (Quiagen) according to the manufacturer's instruction.

Cell culture and transfections. Human embryonic kidney epithelial 293T cells and mouse plasmacytoma MPC11 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. These cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Transfections were performed with Lipofectamine (Gibco-BRL) as instructed by the manufacturer.

Antibodies and immunoblotting. Monoclonal anti-GFP, anti-phospho-Erk, and anti-phospho-threonine and polyclonal anti-Erk antibodies were obtained from Santa-Cruz Biotechnology. Cells were lysed by lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.05% sodium deoxycholate, 50 mM NaF, 200 μM Na_3VO_4, 2 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) for 30 min on ice. Supernatants were collected by centrifugation at 12,000*g* for 10 min at 4 °C, and the protein concentration was determined by the Bradford protein assay. The lysates were boiled in SDS–PAGE sample buffer and separated by SDS–PAGE, transferred to nitrocellulose membranes, probed with antibodies as indicated, and illuminated with the enhanced chemiluminescence (ECL) system (Roche).

Immunoprecipitation. Transfected 293T cells were lysed by cold lysis buffer for 30 min on ice. After centrifugation at 12,000*g* for 10 min at 4 °C, supernatant was collected. The supernatant was precleared by protein A–agarose and incubated with a desired antibody at 4 °C for 2 h. Then, we added 30 μl protein A–agarose and incubated for 16 h at 4 °C. After extensive washing, the immunoprecipitants were resuspended in SDS–PAGE sample buffer and separated by SDS–PAGE.

In vitro kinase assay. Erk kinase assay was performed with immunoprecipitates for anti-Erk antibody. In detail, the cell lysates were subjected to immunoprecipitation with anti-Erk antibody. The immunoprecipitates were washed three times with lysis buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 40 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 200 μM Na_3VO_4, 2 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), and twice with kinase buffer (29 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 1 mM Na_3VO_4), and then resuspended in 20 μl kinase buffer containing 20 μM ATP, 2 μCi [γ-^{32}P]ATP, and substrate. The substrates are 2 μg myelin basic protein and 3 μg *E. coli* purified His-rpS3. For rpS3-associated kinase assay, glutathione–Sepharose 4B bead-immobilized proteins were prepared as described before. The immobilized fusion proteins were incubated overnight with an equivalent amount of MPC11 cell lysates at 4 °C. The coprecipitates were washed three times with lysis buffer and twice with kinase buffer. The kinase reaction was carried out at 30 °C for 30 min by resuspending the beads in 40 μl kinase buffer containing 2 μCi [γ-^{32}P]ATP. The proteins were resolved by SDS–10% PAGE and analyzed by autoradiography.

Results and discussion

Immunocomplex kinase assay revealed the phosphorylation of rpS3

As widely known, many ribosomal proteins are phosphorylated by several kinases and this phosphorylation affects its stability and ribosomal function [23,24]. To find out whether the rpS3 protein interacts with some kinases, we performed a rpS3-associated kinase assay. GST and GST-rpS3 fusion proteins were immobilized on glutathione–Sepharose 4B beads and were incubated with the MPC11 cell lysate. After extensive washing, the complexes bound to the immobilized proteins were subjected to in vitro kinase assay. We then examined the phosphorylation state of the two immobilized proteins. Expectedly, GST-rpS3 protein (Fig. 1A, lane 2) was phosphorylated but GST was not (Fig. 1A, lane 1). Although we did not know whether the phosphorylation occurred on serine/threonine or tyrosine residues of rpS3, we concluded that

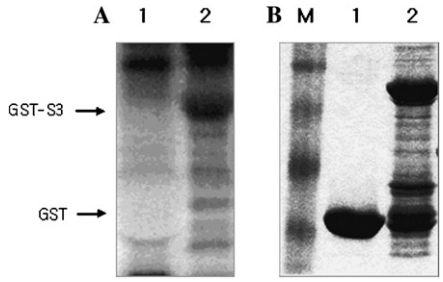

Fig. 1. In vitro rpS3-associated kinase assay. (A) The GST and GST-rpS3 fusion proteins were purified with glutathione–Sepharose 4B beads, and 20 μg of the immobilized proteins was incubated with 2 mg MPC11 cell lysates. After extensive washing, the complexes were subjected to in vitro kinase assay. (B) The immobilized proteins were analyzed on 11% SDS–PAGE gel and detected with Coomassie blue staining. Lane 1, GST; lane 2, GST-S3; and M, molecular marker.

the rpS3 protein was definitely phosphorylated by a certain kinase.

RpS3 has a specific docking site for Erk

Because the rpS3 protein was phosphorylated by an unknown kinase, we made an effort to search for the kinase binding domain on the amino acid residue of the rpS3 protein. Consequently, we found a FXFP motif that is known to mediate a specific interaction with Erk MAPK (Fig. 2A). According to the previous studies [5–7], the FXFP motif is identified as a docking site required for an efficient phosphorylation of a substrate by Erk. Additionally, the substrates of Erk are phosphorylated on a serine or threonine followed by a proline (S/TP) as the minimal consensus sequence—phosphoacceptor motif [26,27]. From this point of view, the rpS3 protein appears to have two putative sites (Thr 42 and Thr 221) that can be candidates as the phosphoacceptor motif for Erk. However, the docking site is generally located downstream from the phosphorylated serines or threonines. Taken together, the Thr 42 residue is the optimal site for the phosphorylation by Erk.

RpS3 is a substrate for Erk but not other MAP kinases in vitro

To determine which MAP kinase phosphorylated rpS3, we performed an immunocomplex kinase assay using His-rpS3 as a substrate. Activities of MAP kinases were measured in immune-complex kinase assays of 293T cell lysates using myelin basic protein as a substrate for the ERK assay, GST-c-Jun$_{1–79}$ for the JNK assay, and GST-ATF2$_{1–109}$ for the p38 MAPK assay. As shown in Fig. 3, the His-rpS3 protein was readily phosphorylated by ERK in vitro, but not by JNK and p38 MAPK. Thus, we concluded that the rpS3 protein is a substrate for Erk.

RpS3 interacts with inactive Erk

Next, we investigated whether the interaction between rpS3 and Erk could actually occur in cells. GFP and GFP-fused rpS3 constructs were transiently transfected into 293T cells, and the transient transfectants were treated with the MEK inhibitor, PD98059 (Fig. 4A). And then, the presence of rpS3 in anti-Erk

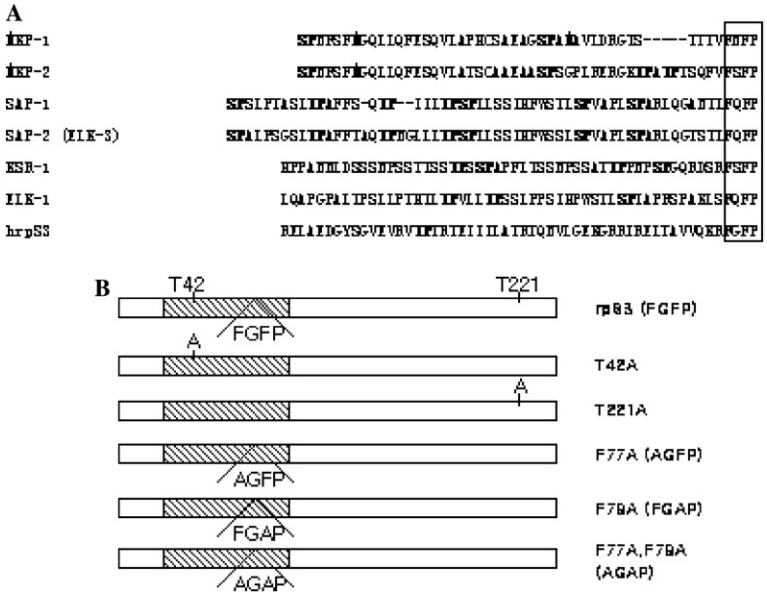

Fig. 2. Sequences of Erk MAPK docking site—FXFP. (A) The box shows the docking sites of substrates for Erk. Ser-Pro and Thr-Pro consensus sites for Erk phosphorylation are in bold. *Abbreviations:* MKP, MAPK phosphatase; SAP, SRF accessory protein; GATA, transcription factors binding to the nucleotide sequence GATA; KSR, kinase suppressor of Ras; ELK, Ets-like transcription factor; and hrpS3, human ribosomal protein S3. (B) Schematic representations of rpS3 wild type and mutants on Erk binding or phosphorylation sites. Hatched area indicates the KH domain.

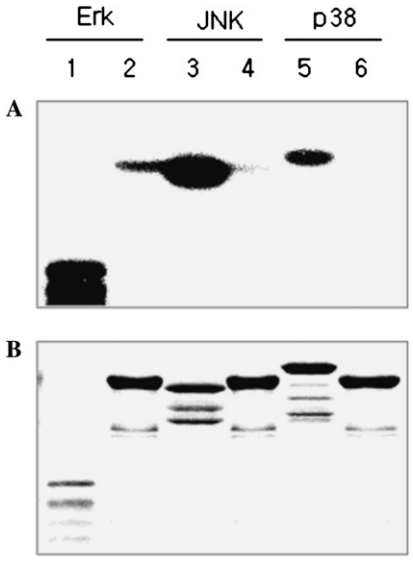

Fig. 3. RpS3 is phosphorylated by Erk but not JNK and p38. (A) 293T cells were unexposed or exposed to UV light (50 J/m^2) and then incubated further for 30 min. Cell lysates were subjected to immunoprecipitation with anti-Erk, anti-JNK, and anti-p38 antibodies. Immunoprecipitates were used in in vitro kinase assay with purified wild-type His-rpS3 (lanes 2, 4, and 6) and with their substrates. Phosphorylation was detected by isotope labeling with [γ-^{32}P]ATP followed by phosphorimaging analysis. (B) MBP, GST-cJun (1–79), GST-ATF (1–109), and the purified His-rpS3 proteins were analyzed on 11% SDS–PAGE gel and were detected with Coomassie blue staining. Lane 1, MBP; lanes 2, 4, and 6, His-rpS3; lane 3, GST-cJun; and lane 5, GST-ATF2.

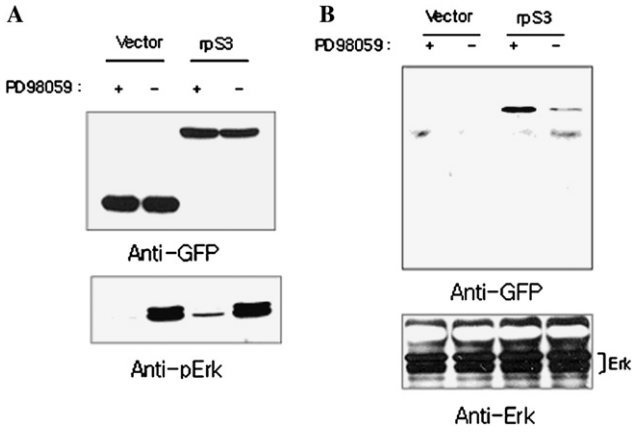

Fig. 4. Activated Erk shows decreased interaction with rpS3. 293T cells were transiently transfected with GFP or GFP-rpS3, and the transfectants were either untreated (−) or treated (+) with 10 μM PD98059 for 1 h. (A) The cell lysates were separated by 11% SDS–PAGE and analyzed by immunoblotting against anti-GFP (upper panel) and anti-phospho Erk (bottom panel) antibodies. (B) The lysates were subjected to immunoprecipitation with anti-Erk antibody. Co-immunoprecipitants were separated by 11% SDS–PAGE and analyzed by immunoblotting against anti-GFP (upper panel) and anti-Erk (bottom panel) antibodies.

immunoprecipitates was analyzed by immunoblotting. As shown in Fig. 4B, Erk interacted with the GFP-fused rpS3 (Fig. 4B, lanes 3 and 4) but not with GFP vector (Fig. 4B, lanes 1 and 2). Also, we examined that the inactive Erk (Fig. 4B, lane 3) interacted with rpS3 more efficiently than the active Erk (Fig. 4B, lane 4), indicating that the interaction between rpS3 with Erk mainly takes place in a normal inactive state but not in an activated state.

Erk phosphorylates on threonine 42 of rpS3 by an interaction with FGFP sequence in vitro

To determine whether the FGFP sequence in rpS3 could play a role in an Erk docking site, we generated three GFP-rpS3 mutants in which the two phenylalanine residues were converted to alanines. In contrast to the wild-type rpS3 and single amino acid mutants (AGFP or FGAP), the double amino acid mutant (AGAP) was not able to be co-precipitated with Erk antibody (Fig. 5A). These data demonstrate that the FGFP site in the rpS3 protein acts as a docking site for Erk MAPK. To confirm this result, we performed an immunocomplex kinase assay with His-tagged mutants. Whereas the two single mutants were less phosphorylated than the wild type, the double mutant was not phosphorylated by Erk at all.

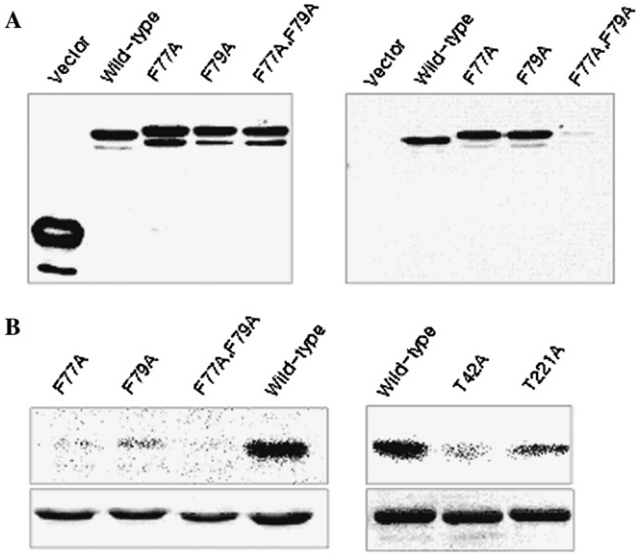

Fig. 5. Erk phosphorylates the threonine 42 of rpS3 via interaction with FXFP sequence. (A) 293T cells were solubilized in a lysis buffer, and the cell lysate was immunoprecipitated with anti-Erk antibody. Immunoprecipitated Erk was used for in vitro kinase assay with purified mutant or wild-type His-rpS3. Phosphorylation was confirmed by isotope labeling with [γ-^{32}P]ATP followed by phosphorimaging analysis (upper panels). The His-purified proteins were analyzed on 11% SDS–PAGE gel and were detected with Coomassie blue staining (bottom panels). (B) 293T cells were transiently transfected with vector, wild type, and mutant rpS3. The cell lysates were separated by 11% SDS–PAGE and analyzed by immunoblotting against anti-GFP (left panel). And the lysates were subjected to immunoprecipitation with anti-Erk antibody. Co-immunoprecipitants were separated by 11% SDS–PAGE and analyzed by immunoblotting against anti-GFP (right panel).

Additionally, the rpS3 protein has two candidate phosphoacceptor motifs that can be phosphorylated by Erk. To identify whether Thr 42 and Thr 221 residues of rpS3 could be phosphorylated by Erk, we generated two mutants of His-rpS3 in which Thr 42 and Thr 221 were substituted to alanine by using site-directed mutagenesis. And the immunocomplex kinase assay was subsequently performed. As shown in Fig. 5B, Erk phosphorylated wild type (Fig. 5B, lane 1) and T221A mutant (Fig. 5B, lane 3), but marginally phosphorylated T42A mutant His-rpS3 (Fig. 5B, lane 2). In conclusion, we found that the Erk kinase interacts with rpS3 via FXFP motif and phosphorylates the Thr 42 residue of rpS3.

Erk phosphorylates on threonine 42 of rpS3 in vivo

To examine whether the Thr 42 phosphorylation of rpS3 in vivo was dependent on the activation of Erk, we converted the two TA mutants into GFP-fused forms. When the mutants and wild-type rpS3 were transfected into 293T cells, the phosphorylation status of the proteins were detected with anti-phospho-threonine antibody in the immunoprecipitation assay. First, we analyzed the phosphorylation state of rpS3 with or without MEK inhibitor PD98059. As shown in Fig. 6A, the MEK inhibitor PD98059 induced the phosphorylation of the protein more (Fig. 6A, lane 4, bottom panel). Second, we also analyzed the phosphorylation status of mutant rpS3. In the immunoprecipitation assay, the phosphorylation signal was not detected in the T42A mutant (Fig. 6B, lane 3, bottom panel) but it was detected in the wild type and T221A mutant (Fig. 6B, lanes 2 and 4, bottom panel). Thus, we propose that the Erk activation is responsible for the phosphorylation of rpS3 at Thr 42 in vivo. Considering all the data collectively, the rpS3 protein appears to interact with inactive Erk in normal state and the interaction elicits the phosphorylation of rpS3 when Erk is activated, and then the two proteins are dissociated.

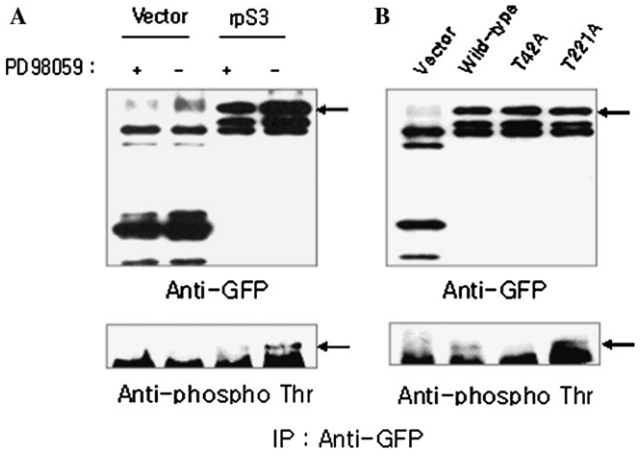

Fig. 6. Activated Erk phosphorylates the threonine 42 of rpS3 in vivo. (A) 293T cells were transiently transfected with GFP or GFP-rpS3, and the transfectants were either untreated (−) or treated (+) with 10 μM PD98059 for 1 h. The cell lysates were subjected to immunoprecipitation with anti-GFP antibody. (B) 293T cells were transiently transfected with GFP, GFP-rpS3, GFP-T42A, and GFP-T221A. The cell lysates were subjected to immunoprecipitation with anti-GFP antibody. The immunoprecipitants were separated by 10% or 8% SDS–PAGE and analyzed by immunoblotting against anti-GFP (upper panel) and anti-phospho-threonine (bottom panel) antibodies. The arrows show the positions of their respective GFP-rpS3 forms.

Because ribosome biogenesis includes the expression of many rRNA genes and the synthesis of many ribosomal proteins and assembly factors while consuming up to 80% of the energy of a proliferating cell, ribosome assembly must therefore be tightly regulated for the economy of the cell and be rapidly responsive to various environmental and intracellular growth conditions [28]. Furthermore, according to the previous data [29], deletion of the rpS3 gene in yeasts results in the lethal phenotype. This fact suggests that the rpS3 protein plays an important role in the ribosome assembly or biogenesis. Here, we showed that the phosphorylation of rpS3 resulted from the interaction with Erk kinase. We also propose a possibility that the phosphorylation status of rpS3 affects the heterogeneity of ribosome [30] existing in two forms in which the rpS3 is phosphorylated or not.

Acknowledgments

This work was supported in part by FPR05C2-390 Proteomics Frontier Science Grant and KOSEF Grant RO1-2005-000-10798-0 from Korean Ministry of Science and Technology.

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