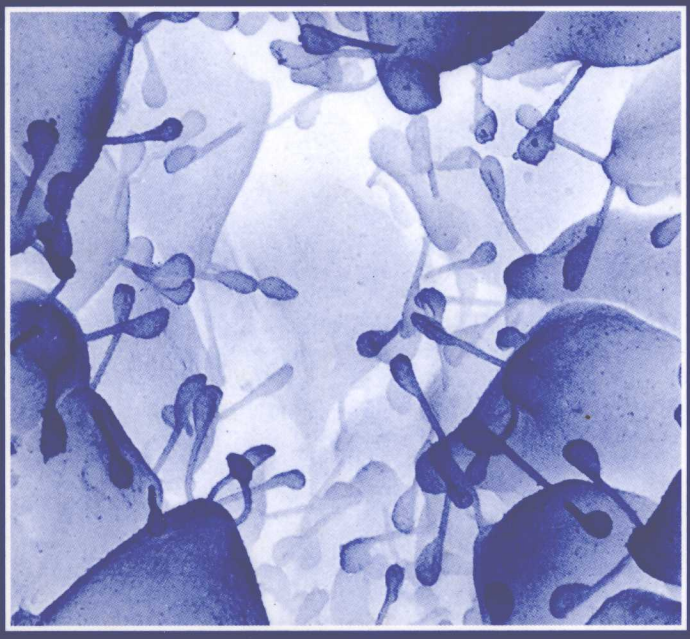
INTERNATIONAL REVIEW OF CELL AND MOLECULAR BIOLOGY

Edited by
Kwang W. Jeon



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Contents

CHAPTER ONE

PHYSIOLOGICAL ROLES OF RIBOSOMAL PROTEIN S6: ONE OF ITS KIND

Oded Meyuhas*

Contents

Abstract

The phosphorylation of ribosomal protein S6 (rpS6), which occurs in response to a wide variety of stimuli on five evolutionarily conserved serine residues, has attracted much attention since its discovery more than three decades ago. However, despite a large body of information on the respective kinases and the signal transduction pathways, the role of this phosphorylation remained obscure. It is only recent that targeting the genes encoding rpS6, the phosphorylatable serine residues or the respective kinases that the unique role of rpS6 and its posttranslational modification have started to be elucidated. This review focuses primarily on the critical role of rpS6 for mouse development, the

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pathways that transduce various signals into rpS6 phosphorylation, and the physiological functions of this modification. The mechanism(s) underlying the diverse effects of rpS6 phosphorylation on cellular and organismal physiology has yet to be determined. However, a model emerging from the currently available data suggests that rpS6 phosphorylation operates, at least partly, by counteracting positive signals simultaneously induced by rpS6 kinase, and thus might be involved in fine-tuning of the cellular response to these signals.

Key Words: Ribosomal protein S6, S6 kinase, RSK, mTOR, Protein synthesis, TOP mRNAs, Cell size, Cell proliferation, Glucose homeostasis.

1. Introduction

The higher eukaryotic ribosomes are composed of two subunits that are designated as 40S (small) and 60S (large) subunits. The mammalian 40S subunit is composed of a single RNA molecule, 18S ribosomal (r) RNA, and 33 proteins, whereas the 60S subunit has three RNA molecules: 5S, 5.8S, and 28S rRNAs, and 46 proteins (Wool *et al.*, 1996). Of all ribosomal proteins, it is ribosomal protein S6 (rpS6) that has attracted much attention, since it is the first, and was for many years the only one, that has been shown to undergo inducible phosphorylation.

The ribosome biogenesis takes place in the nucleolus and starts with the synthesis of 5S and 45S pre-rRNA by distinct RNA polymerases and requires the import of ribosomal proteins from the cytoplasm. A complex pathway that involves both endo- and exonucleolytic digestions enables the release of mature rRNAs from the pre-rRNA. Concomitantly, rRNAs are extensively modified and bound by the ribosomal proteins before the assembled pre-40S and pre-60S subunits are exported separately to the cytoplasm (Fromont-Racine *et al.*, 2003; Zemp and Kutay, 2007). High-resolution cytological analysis has recently disclosed the fate of rpS6 from its biosynthesis site in the cytoplasm to the pre-40S subunit. Thus, rpS6 enters the nucleus of HeLa cells, reaches, via Cajal bodies, the nucleolus, where it is assembled with other proteins and rRNA into pre-40S subunit. The latter is then released to the nucleoplasm prior to its export through the nuclear pores to the cytoplasm (Cisterna *et al.*, 2006). Interestingly, the nuclear import, as well as the nucleolar localization of human rpS6 and yeast rpS6A, rely on motifs, whose number, nature, and position are evolutionary conserved (Lipsius *et al.*, 2005; Schmidt *et al.*, 1995).

The phosphorylation of rpS6 has attracted much attention in numerous labs since its discovery in 1974 (Gressner and Wool, 1974b). However, it is only recently that the role of rpS6 and its posttranslational modification has started being disclosed by genetic targeting of the *rpS6* gene and of the

respective kinases. Hence, this review includes a brief account on the evolutionary conservation of rpS6, as well as the enzymes that conduct, and the cues that affect its phosphorylation, and a comprehensive discussion on the critical role of rpS6 for mouse development, pathways that transduce various signals into rpS6 phosphorylation, and the physiological role of this modification.

2. General Background

2.1. Evolutionary conservation of rpS6

rpS6 is an evolutionary conserved protein that spans 236–253 residues in species as remote as yeast, plants, invertebrates, and vertebrates (Fig. 1.1), yet no homology with any ribosomal protein in *E. coli* or archebacteria has

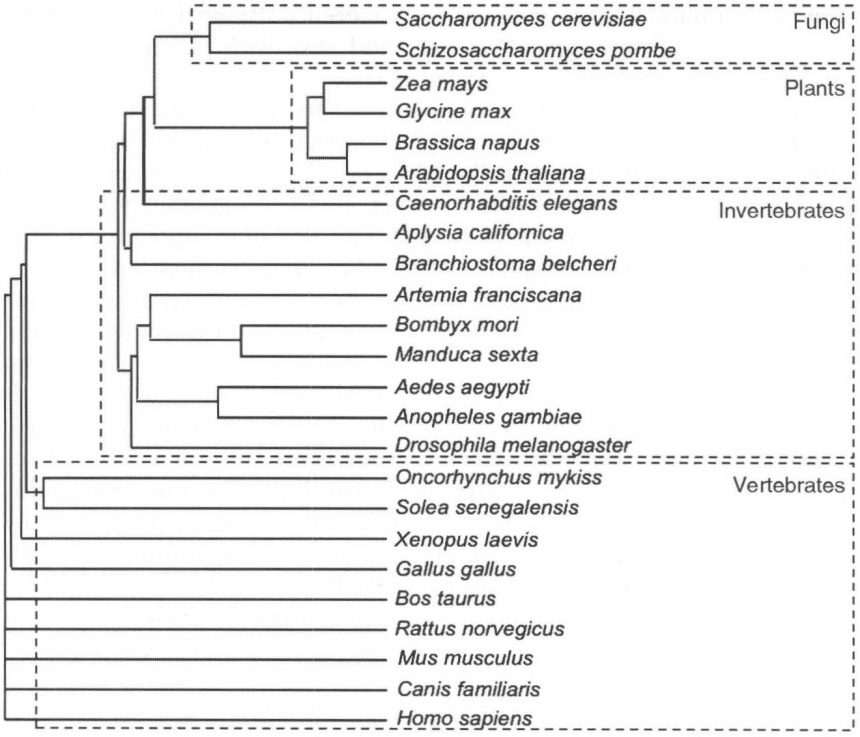

Figure 1.1 The phylogenetic tree of sequences of rpS6 orthologs in different eukaryotic species. Alignments and the tree were generated using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/index.html).

been detected (Wool *et al.*, 1996). Interestingly, rpS6 with C-terminal extensions, ranging in length from 81 to 190 amino acids and enriched for lysine and alanine, is widespread among the Culicomorpha (an infraorder of Nematocera that includes mosquitoes and black flies) (Fallon and Li, 2007). The C-terminal extensions on rpS6 from the mosquitoes *Aedes aegypti* and *Aedes albopictus* are 42–49% homologous with histone H1 proteins from other multicellular organisms (Hernandez *et al.*, 2003).

2.2. rpS6 is an indispensable ribosomal protein

The role of rpS6 was first addressed by conditional knockout of the respective gene in adult mouse liver (Volarevic *et al.*, 2000). Hepatocytes that lacked *rpS6* gene failed to synthesize the 40S ribosomal subunit and consequently to proliferate following partial hepatectomy. This failure to progress through the cell cycle correlated with a block in expression of cyclin E gene. Nonetheless, the expression of *rpS6* gene was not required for liver growth when starved mice were refed. Moreover, the relative engagement of liver ribosomes in translation, as exemplified by their polysomal association, was indistinguishable between rpS6-containing and -lacking hypertrophying livers (Volarevic *et al.*, 2000).

The critical role of rpS6 is not confined to the regenerating liver, as thymus-specific knockout of *rpS6* gene, but not conditional deletion of one allele, had devastating effect on the gland development (Sulic *et al.*, 2005). rpS6 heterozygosity ($rpS6^{wt/del}$), however, had a remarkable effect on the number of mature T cells in peripheral lymphoid organs (spleen and lymph nodes). The deficiency of one *rpS6* allele led to a proportional diminution in the abundance of rpS6 and ribosome contents in purified $rpS6^{wt/del}$ T cells, yet with no effect on their total protein content or their ability to undergo normal stimulated cell growth (Sulic *et al.*, 2005). Likewise, 30–50% reduction in rpS6 content of HeLa cells by siRNA only mildly affected global protein synthesis (Montgomery *et al.*, 2006). Nevertheless, while wild-type T cells progressed *in vitro* through several divisions upon mitogenic stimulation, their $rpS6^{wt/del}$ counterparts failed to proliferate, as a result of a block at the G1/S checkpoint of the cell cycle, and partially due to increased apoptosis. Interestingly, deletion of both *p53* alleles almost completely resumed the proliferative capacity of stimulated $rpS6^{wt/del}$ T cells. These observations strongly support the notion that impaired ribosome biogenesis, associated with rpS6 deficiency, activates a p53-dependent checkpoint to eliminate defective T cells (Sulic *et al.*, 2005).

$rpS6^{wt/del}$ embryos died during gastrulation at day 8.5. However, already at day 6.5, their cells failed to show dephosphorylation and activation of Cdk1 and to enter mitosis. Moreover, the embryonal death was preceded by induced apoptosis. The fact that *p53* gene knockout enabled $rpS6^{wt/del}$ embryo to develop past gastrulation stage, suggests that rpS6 heterozygosity

triggers a p53-mediated checkpoint during gastrulation. Interestingly, ribosome biogenesis is defective in *rpS6*$^{wt/del}$/*P53*$^{-/-}$ embryo, as well as in the corresponding mouse embryo fibroblasts (MEFs). However, while neither cell cycle progression nor cell growth is impaired in *rpS6*$^{wt/del}$/*P53*$^{-/-}$ MEFs, compromised cell proliferation was observed in the liver from *rpS6*$^{wt/del}$/*P53*$^{-/-}$ embryo. This decreased in hepatic proliferation might be explained by the relative deficiency of cyclins D1 and D3, observed in this organ (Panic *et al.*, 2006).

Lesions in *Drosophila rpS6* gene expression, due to insertion of P element upstream of the transcription initiation site, had a mixed response: hyperplasia of lymphglands on the one hand and growth inhibition of most larval organs on the other hand (Stewart and Denell, 1993; Watson *et al.*, 1992).

The critical role of rpS6 is underscored by the fact that it is the only ribosomal protein, for which it has been shown, so far, that heterozygosity leads to early embryonal lethality (Panic *et al.*, 2006). Thus, *rpL24*$^{-/-}$ mice die before E9.5, yet the heterozygotes are viable, even though exhibiting dysmorphic feature and reduced somatic growth (Oliver *et al.*, 2004). Similarly, *rpS19* null mutation is lethal prior to implantation, whereas rpS19$^{+/-}$ mice have normal growth and organ development (Matsson *et al.*, 2004). Unlike rpS6, rpL29 seems to be a dispensable ribosomal protein, as mice with disruption of both *rpL29* alleles suffer of global growth deficiency, yet they are viable (Kirn-Safran *et al.*, 2007). Similarly, it has recently been shown that efficient depletion (2% residual activity) of rpL13a in human monocytic cells by short hairpin RNA had no significant effect on global protein synthesis, translational fidelity, or cell proliferation. These results suggest, therefore, that rpL13a is dispensable for canonical ribosome function (Chaudhuri *et al.*, 2007).

3. Phosphorylation of rpS6

3.1. Stimuli inducing rpS6 phosphorylation

A pioneer study conducted by David Kabat has shown that a 33-kDa protein, termed F protein, which resided in the small ribosomal subunit undergoes phosphorylation in rabbit reticulocytes (Kabat, 1970). Later, it has been identified as rpS6, and that it is the only ribosomal protein that undergoes phosphorylation during rat liver regeneration (Gressner and Wool, 1974b). A flood of subsequent reports has demonstrated that rpS6 is subject to phosphorylation in response to numerous physiological, pathological, and pharmacological stimuli (see Table 1.1). Notably, this modification can be detected in both the cytosol and the nucleus (Pende *et al.*, 2004).

Table 1.1 rpS6 is phosphorylated by multiple

Treatment	Organism/cell	Phosphorylation	References
(A) Mitogenic stimulation			
(a) Liver regeneration	Rat	⇑	Gressner and Wool (1974b)
(b) Growth factors and cytokines			
(1) Serum, IGF	Chick embryo fibroblasts	⇑	Haselbacher *et al.*, (1979)
(2) EGF	Mouse Swiss 3T3 cells	⇑	Thomas *et al.*, (1982)
(3) NGF	Rat PC12 cells	⇑	Halegoua and Patrick (1980)
(4) PDGF	Mouse Swiss 3T3 cells	⇑	Nishimura and Deuel (1983)
(5) Interleukin 2	Mouse T lymphocytes	⇑	Evans and Farrar (1987)
(B) Hormones			
(a) Insulin	Mouse 3T3-L1 cells	⇑	Smith *et al.*, (1979)
(b) Glucagon	Rat liver	⇑	Gressner and Wool (1976)
(c) Progesterone	*Xenopus* oocyte	⇑	Nielsen *et al.*, (1982)
(d) Estrogen	Rooster hepatocytes	⇑	Cochrane and Deeley (1984)
(e) PTTH	Tobacco hornworm	⇑	Song and Gilbert (1997)
(f) Juvenile hormone	Flesh-fly	⇑	Itoh *et al.*, (1987)
(C) Nutrients			
(a) Amino acids	Human HEK293 cells	⇑	Tang *et al.*, (2001)
(b) Leucine	Rat L6 myoblasts	⇑	Kimball *et al.*, (1999)
(c) Glucose	Mouse MIN6 β-cells	⇑	Gleason *et al.*, (2007)
(D) Lipid compounds			
(a) Diacyl glycerol	Mouse T lymphocytes	⇑	Evans and Farrar (1987)
(b) Prostaglandin $F_{2\alpha}$	Mouse Swiss 3T3 cells	⇑	Thomas *et al.*, (1982)
(E) Viral infection			
(a) Vaccinia virus	Human HeLa cells	⇑	Kaerlein and Horak (1976)

Table 1.1 *(continued)*

Treatment	Organism/cell	Phosphorylation	References
(b) Pseudorabies	Hamster fibroblasts	⇑	Kennedy *et al.*, (1981)
(c) Polyoma virus	Hamster fibroblasts	⇑	Kennedy and Leader (1981)
(d) Simian virus	Hamster fibroblasts	⇑	Kennedy and Leader (1981)
(e) Avian sarcoma virus	Chick embryo fibroblasts	⇑	Decker (1981)
(f) AMLV	Mouse NIH 3T3 fibroblasts	⇑	Maller *et al.*, (1985)
(g) Alphavirus	Human HEK293	⇓	Montgomery *et al.*, (2006)
(F) Stresses			
(a) Hypoxia	Human HEK293 cells	⇓	Arsham *et al.*, (2003)
	Maize root tips	⇓	Williams *et al.*, (2003)
(b) Heat shock	*Drosophila*	⇓	Glover (1982)
	Human HeLa cells	⇓	Kennedy *et al.*, (1984)
	Tomato cell suspension	⇓	Scharf and Nover (1982)
(c) Hyperosmolarity	Mouse myeloma	⇓	Kruppa and Clemens (1984)
(G) Pharmacological agents			
(a) Translation inhibitors			
(1) Cycloheximide	Rat liver	⇑	Gressner and Wool (1974a)
(2) Puromycin	Rat liver	⇑	Gressner and Wool (1974a)
(b) Transcription inhibitors			
(1) d-Galactosamine	Rat liver	⇑	Gressner and Greiling (1977)
(2) DRB	Human HeLa cells	⇑	Duncan and McConkey (1984)
(c) Energy depletion			
(1) 2-Deoxyglucose	Human HEK293	⇓	Inoki *et al.*, (2003)

(continued)

Table 1.1 (*continued*)

Treatment	Organism/cell	Phosphorylation	References
(2) 5-Thioglucose	Mouse embryo fibroblasts	⇓	Hahn-Windgassen *et al.*, (2005)
(d) Phorbol esters	Rat hepatoma cells	⇑	Trevillyan *et al.*, (1984)

Notes: ⇑, increase; ⇓, decrease; AMLV, Abelson murine leukemia virus; DRB, dichlororibofuranozyl benzimidazole; EGF, epidermal growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PTTH, prothoracicotropic hormone.

3.1.1. Evolutionary conservation of rpS6 phosphorylation sites

The phosphorylation sites in rpS6 in mammals and *Xenopus laevis* have been mapped to five clustered residues: S^{235}, S^{236}, S^{240}, S^{244}, and S^{247} (Bandi *et al.*, 1993; Krieg *et al.*, 1988; Wettenhall *et al.*, 1992), whose location at the carboxy terminus of higher eukaryotes is evolutionarily conserved (Table 1.2). It has been proposed that phosphorylation progresses in an ordered fashion, with Ser236 as the primary phosphorylation site (Flotow and Thomas, 1992; Wettenhall *et al.*, 1992). A similar organization of phosphorylation sites, relative to the carboxy terminus was described for *Drosophila melanogaster* rpS6 (Radimerski *et al.*, 2000 and Table 1.2).

Maize (*Zea mays*) rpS6 appears to be encoded by two genes and the resulting proteins are identical except for two amino acid substitutions. The relative location of the five phosphorylation sites is comparable with that of vertebrate rpS6, yet this set of sites is not confined to serines, as it include also a threonine residue (Williams *et al.*, 2003 and Table 1.2).

The first report on the phosphorylation of rpS6 (S10 according to an older nomenclature) in *Saccharomyces cerevisiae* lagged behind that of its mammalian counterpart (Hebert *et al.*, 1977). Yeast rpS6 is phosphorylated after transfer of a stationary culture to fresh nutrient medium, as well as at an early stage of germination, and as in other eukaryotes, the protein is dephosphorylated during heat shock (Jakubowicz, 1985; Szyszka and Gasior, 1984). However, yeast rpS6, unlike higher eukaryotes, bears only two phosphorylatable serine residues (Ser232 and Ser233) that correspond to Ser235 and Ser236 in the mammalian protein.

3.1.2. S6 kinase (S6K1 and S6K2)

Characterization of an S6 kinase at a molecular level was first achieved in *Xenopus* oocytes wherein the dominant form of S6 kinase detected after mitogenic stimulation had been purified as a 90-kDa polypeptide (Erikson and Maller, 1985), later termed as p90 ribosomal protein S6 kinase (RSK, also known as $p90^{RSK}$). Purification of the avian and mammalian major