Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis

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The regulated phosphorylation of ribosomal protein (rp) S6 has attracted much attention since its discovery in 1974, yet its physiological role has remained obscure. To directly address this issue, we have established viable and fertile knock-in mice, whose rpS6 contains alanine substitutions at all five phosphorylatable serine residues (rpS6P−/−). Here we show that contrary to the widely accepted model, this mutation does not affect the translational control of TOP mRNAs. rpS6P−/− mouse embryonic fibroblasts (MEFs) display an increased rate of protein synthesis and accelerated cell division, and they are significantly smaller than rpS6P+/+ MEFs. This small size reflects a growth defect, rather than a by-product of their faster cell division. Moreover, the size of rpS6P−/− MEFs, unlike wild-type MEFs, is not further decreased upon rapamycin treatment, implying that the rpS6 is a critical downstream effector of mTOR in regulation of cell size. The small cell phenotype is not confined to embryonal cells, as it also selectively characterizes pancreatic β-cells in adult rpS6P−/− mice. These mice suffer from diminished levels of pancreatic insulin, hypoinsulinemia, and impaired glucose tolerance.

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Phosphorylation of rpS6 was first discovered during liver regeneration [Gressner and Wool 1974], and later on in response to numerous agents and alterations in the cellular milieu (for review, see Chan and Wool 1988). The phosphorylation sites in rpS6 have been mapped to five clustered residues—Ser235, Ser236, Ser240, Ser244, and Ser247—which are located at the C terminus [Krieg et al. 1988] and are conserved from Drosophila to mammals (Fumagalli and Thomas 2000). This phosphorylation has attracted much attention due to its temporal correlation with the initiation of protein synthesis, and the suggestion that ribosomes with the highest proportion of phosphorylated rpS6 have a selective advantage in mobilization into polysomes (for review, see Fumagalli and Thomas 2000). Phosphorylation of rpS6 has a selective advantage in mobilization into polysomes (for review, see Fumagalli and Thomas 2000). Phosphorylation of rpS6 is carried out in Drosophila by a single kinase, dS6K, whereas in mice the phosphorylation is performed by two closely related kinases, S6K1 and S6K2 [Fumagalli and Thomas 2000]. Analysis of S6 phosphorylation in mouse cells deficient of either of these kinases suggests that both are required for full S6 phosphorylation, with predominance of S6K2. However, phosphorylation of rpS6 can still be detected at Ser235 and Ser236 in cells lacking both these kinases (S6K1−/−/S6K2−/−), conceivably by mitogen-activated protein kinase [Pende et al. 2004]. S6Ks have been implicated as important positive regulators of cell and body size. Thus, most dS6K-null Drosophila exhibit embryonic lethality, with the few surviving adults having a severely reduced body size, due to a decrease in cell size rather than a decrease in cell number [Montagne et al. 1999]. S6K1−/− mice are significantly smaller at birth [Shima et al. 1998], with a reduced size of adult pancreatic β-cells, diminished levels of circulating insulin, and a mild glucose intolerance [Pende et al. 2000]. S6K2−/− mice are not smaller than the wild-type mice, whereas embryonic and post-natal growth of S6K1−/−/S6K2−/− of surviving mice is similar to that of S6K1−/− mice [Pende et al. 2004]. Nevertheless, despite the increasing list of physiological roles assigned to S6K activity, the involvement of its best-characterized substrate, rpS6, as well as its other assigned substrates [de Groot et al. 1994; Wilson et al. 2000; Harada et al. 2001; Wang et al. 2001; Harrington et al. 2004; Raught et al. 2004; Richardson et al. 2004], in any of these functions has remained unknown.
A prevailing model has implicated rpS6 phosphorylation in translational control of TOP mRNAs. These mRNAs are characterized by an oligopyrimidine tract at their 5’ terminus (5’-TOP) and encode various proteins associated with the function or the assembly of the translational apparatus (Meyuhas 2000). The translation of these mRNAs is selectively activated when resting cells are induced to grow (increase in their mass) or to proliferate, or when amino acid-starved cells are refed (Tang et al. 2001; Stolovich et al. 2002). The 5’-TOP motif comprises a critical translational cis-regulatory element in these mRNAs (Levy et al. 1991).

The temporal relationship between the translational activation of TOP mRNAs and the activation of S6K and the phosphorylation of its substrate, rpS6, led to a model that related translational efficiency of TOP mRNAs to rpS6 phosphorylation (Fumagalli and Thomas 2000). However, recent reports have shown that TOP mRNAs are subject to normal translational control in S6K1−/− embryonic stem (ES) cells, whose rpS6 is constitutively unphosphorylated (Tang et al. 2001; Stolovich et al. 2002). Consequently, the physiological role of rpS6 phosphorylation has once again become obscure. We set out, therefore, to elucidate the role rpS6 phosphorylation by serine-to-alanine substitution of all five phosphorylatable residues in mouse rpS6. To this end, we have knocked in a mutated rpS6 gene by homologous recombination and have characterized the phenotype of the resulting homozygous mice.

Results

Generation of rpS6P−/− knock-in mice

Replacement of serine residues 235, 236, 240, 244, and 247 with alanines was carried out by site-directed mutagenesis, which also created AatII and EcoRV sites 5’ and 3’, respectively, to the mutated serine codons. A targeting vector containing these alterations was electroporated into ES cells, and three out of five clones with homologous recombination at the rpS6 locus were selected for establishing chimeric mice, which transmitted the mutant allele. The F2 generation was genotyped by Southern blot and PCR analyses (Fig. 1B,C). The frequency of rpS6P+/+, rpS6P+/-, rpS6P−/− was 1:2:1 (data not shown). RT–PCR analysis of total liver RNA from

![Figure 1](https://genesdev.cshlp.org)
rpS6P+/+ and rpS6P−/− mice demonstrated expression of just the rpS6P−/− allele (data not shown). The mutant rpS6 allele was expressed at least as well as the wild-type allele in heterozygous ES cells, as exemplified by RT-PCR analysis of the respective two transcripts [RT-PCR in Fig. 1D]. Moreover, the presence of the neo gene immediately downstream of the rpS6 gene, as well as the substitution of nucleotides and amino acids, did not impede the abundance of the corresponding mRNA and protein, respectively, in differentiated homozygous mouse embryo fibroblasts (MEFs) [see Northern and Western in Fig. 1D]. Western blot analysis, using antiphospho-Ser235/236 or antiphospho-Ser240/244 antibodies, failed to detect any phosphorylation of these sites in rpS6 in livers of untreated or partially hepatectomized rpS6P−/− mice. In contrast, phosphorylation of the respective sites in rpS6 from rpS6P+/+ mice was readily detected before the operation, and was considerably enhanced in the regenerating liver (Fig. 1E). Moreover, metabolic labeling of rpS6 with [32P]orthophosphate demonstrated that rpS6 is not phosphorylated on other sites in rpS6P−/− MEFs [Fig. 1F]. Taken together, these data clearly demonstrate that all phosphorylatable serine residues in rpS6 are absent in rpS6P−/− mice. Interestingly, rpS6P−/− mice appeared normal from their birth until adulthood and were largely indistinguishable from their wild-type or heterozygous littermates in weight, appearance, development, and fertility.

rpS6 phosphorylation is dispensable for translational control of TOP mRNAs

Early correlative studies led to the assumption that rpS6 phosphorylation is involved in translational control of a subset of mRNAs, TOP mRNA, rather than in global protein synthesis [Jefferies et al. 1994]. However, later experiments conducted with cultured cells have clearly shown that translational activation of TOP mRNAs does not require rpS6 phosphorylation [Tang et al. 2001; Stolovich et al. 2002]. Nonetheless, it has recently been shown that rpS6 is still phosphorylated at Ser235 and/or Ser236 in hepatocytes from S6K1−/−/S6K2−− mice, by mitogen-activated protein kinase, and that the translation of their TOP mRNAs is normally regulated [Pende et al. 2004]. To examine whether this residual phosphorylation of rpS6 is essential and/or sufficient for translational control of TOP mRNAs, we examined the translational behavior of these mRNAs in rpS6P−/− mice and MEFs. Figure 2A shows that a typical TOP mRNA encoding rpL32 was translationally repressed in livers of both rpS6P+/+ and rpS6P−/− mice, as judged by its low association with polysomes. Partial hepatectomy, however, induced similar translational activation (recruitment into polysomes) of rpL32 mRNA in the regenerating liver of both genotypes of mice, even though rpS6 is phosphorylated only in the wild-type liver (Fig. 1F). A non-TOP mRNA, encoding superoxide dismutase (SOD), is translationally active before and after the operation (mostly associated with polysomes) in both rpS6P+/+ and rpS6P−/− mice [Fig. 2A]. The translation of rpL32 mRNA was more repressed in serum-starved rpS6P−/− MEFs than in rpS6P+/+ MEFs, yet it underwent a more pronounced translational activation upon serum refeeding in the mutant relative to the wild-type cells [1.9-fold vs. 1.6-fold, respectively] [Fig. 2B]. It can be argued that the lack of an inhibitory effect of the mutation on the translational activation of rpL32 mRNA might reflect an exceptional behavior of this mRNA, and/or poor resolution of its polysomal association, due to partitioning of the gradient into just two fractions. Hence, we set out to monitor the polysomal distribution of two other TOP mRNAs,
encoding rpS6 and rpS16, in gradients divided into 12 fractions (Fig. 2C). The results demonstrate that not only was the translational efficiency of these mRNAs not impaired in rpS6P−/− cells, it was slightly augmented, as can be judged by the shift of the peaks toward heavier fractions (bottom-wise). Notably, the difference in the location of the peak fractions between rpS6P−/− and rpS6P+/+ cells reflects the size difference of their coding sequence (145 and 249 codons, respectively). Taken together, these results clearly disprove any causal relationship between rpS6 phosphorylation and translational activation of TOP mRNAs.

rpS6 phosphorylation and global protein synthesis

A previous study has demonstrated that rpS6 is located near the mRNA/tRNA-binding site, at the interface between the small and the large subunit [Nygard and Nika 1982]. This observation, together with the notion that phosphorylated 40S ribosomal subunits form initiation complexes more efficiently [Duncan and McConkey 1982], led to the hypothesis that rpS6 phosphorylation might be involved in control of initiation of translation [Thomas et al. 1982]. In order to directly examine the involvement of rpS6 phosphorylation in the control of global protein synthesis, we monitored the proportion of ribosomes that sedimented in the polysome region upon sucrose gradient centrifugation. Figure 3A shows that 74% ± 3% (n = 4) and 75% ± 2% (n = 4) of ribosomal particles from livers of untreated rpS6P+/+ and rpS6P−/− mice, respectively, were engaged in polysomes, whereas these figures were 71% ± 2% (n = 4) and 74% ± 1% (n = 4), respectively, in regenerating livers. These results clearly indicate that elimination of all phosphorylatable sites in rpS6 does not impair the engagement of ribosomes in polysomes.

Next, we measured the rate of protein synthesis in rpS6P+/+ and rpS6P−/− cells by monitoring the incorporation of [35S]methionine and [35S]cysteine into total cytoplasmic proteins. Surprisingly, not only was the incorporation not abrogated in rpS6P−/− cells, it was stimulated by a factor of 2.5 (Fig. 3B). One plausible explanation for the enhanced incorporation of radioactive amino acids into proteins in rpS6P−/− MEFs is a smaller pool of free amino acids and consequently a higher specific activity of the labeled amino acids in these cells, rather than the result of accelerated protein synthesis. To distinguish between these two possibilities, we monitored protein accumulation in all cell types. Figure 3C shows that steady-state accumulation of proteins in rpS6P−/− MEFs is 1.4-fold faster than in wild-type MEFs, thus underscoring the superiority of the former in protein synthesis.

To examine whether the enhanced protein synthesis in rpS6P−/− cells results from accelerated peptide chain elongation, we determined the ribosome half-transit time [Fan and Penman 1970]. This was performed by measuring the kinetics of radioactive amino acid incorporation into total protein in post-mitochondrial supernatant (PMS) and into completed polypeptides released from the ribosome in post-ribosomal supernatant (PRS).

Figure 3. The effect of phosphorylatable serine deficiency in rpS6 on global protein synthesis in liver and MEFs. [A] rpS6P+/+ and rpS6P−/− male mice underwent partial hepatectomy, and 23 h later cytoplasmic extracts were prepared from the remaining liver (Partial hepatectomy) or from livers of untreated mice (Control). These extracts were size-fractionated by centrifugation through sucrose gradients, and the absorbance of polysomes and subpolysomal particles was continuously monitored at 260 nm. (M) Monosomes; (60) 60S subunits; (40S) 40S subunit. The vertical dashed line separates the polysomal fraction [left] and the subpolysomal fraction [right]. The areas under the curve within these fractions were estimated by weighing paper cutouts of the profiles. The proportion of the area in the polysomal fraction has been referred to as the percentage of ribosomes engaged in translation. The results are presented as a mean ± SEM (n = 3). (B) rpS6P+/+ and rpS6P−/− MEFs were pulse-labeled with [35S]methionine and [35S]cysteine, and the protein synthesis was measured and presented as described in Materials and Methods. Protein synthesis at each time point for each cell type represents a mean ± SEM (n = 4). (C) Growing rpS6P+/+ and rpS6P−/− MEFs [passage 2] were harvested at time 0 and 24 h later. Cells were lysed, and protein concentration in extracts was determined as described in Materials and Methods. The protein content at time 0 was arbitrarily set at 1, and that of 24 h was normalized to this value. Results are presented as a mean ± SEM (n = 3). (**) P < 0.001 versus rpS6P−/− MEFs. (D) The ribosome half-transit time in rpS6P+/+ and rpS6P−/− MEFs was determined as described in Materials and Methods. Incorporation of [3H]leucine into total protein within the PMS and PRS was obtained by linear regression analysis. The radioactivity at each time point is presented as a mean ± SEM (n = 3 and 5 for rpS6P+/+ and rpS6P−/− MEFs, respectively).

The average half-transit time was determined from the displacement in time between the two lines corresponding to the PMS and PRS data plotted as a function of time [Fig. 3D], and was calculated to be 76 and 65 sec, for rpS6P+/+ and rpS6P−/− MEFs, respectively. The small decrease (14%) in the half-transit time of the mutant ribosomes, although reflecting a slight increase in the elongation rate, cannot account for the 1.4-fold increase in the rate of protein accumulation in rpS6P−/− MEFs [Fig. 3C].
Taken together, the experiments with whole animals and MEFs indicate that phosphorylatable serine residues in rpS6 are dispensable for global protein synthesis in the liver and are inhibitory in MEFs. The mechanism underlying the enhanced protein synthesis in rpS6P−/− MEFs involves mostly the initiation step, yet its exact nature has yet to be determined.

rpS6 phosphorylation sites are a determinant of cell size

Drosophila cells lacking dS6K have been shown to be smaller than wild-type cells [Montagne et al. 1999]. However, the relevant effector that is involved in the regulation of cell size has remained obscure. We therefore, set out to examine whether the phosphorylatable serine residues in rpS6 are a determinant of cell size. To this end, rpS6P−/− and rpS6P+/+ MEFs were analyzed for cell size, using the parameter mean forward scatter height (FSC-H), which is a measure of relative cell size. Figure 4A shows that rpS6P−/− MEFs are 24% smaller than rpS6P+/+ MEFs. Interleukin 7 (IL-7)-dependent cells, derived from fetal livers at day 14 of gestation and maintained in culture for 10 d, were subjected to flow cytometry. This analysis demonstrated that rpS6P−/− IL-7-dependent cells were 19% smaller than wild-type cells (n = 4 for each genotype) [data not shown]. Apparently, the smaller size of both these types of cells is not reflected in the size of newborn rpS6−/− mice, whose weights between day 1 and day 32 are indistinguishable from that of their wild-type littermates [Fig. 4B]. One plausible explanation for the lack of impact of the size of these cells on the birth weight of the respective mice is a faster proliferation, which compensates for the smaller size of these cells. This possibility accords with the higher rate of protein accumulation observed in rpS6P−/− MEFs [Fig. 3C]. Indeed, the population doubling time (tP) of rpS6−/− MEFs is significantly shorter than that of rpS6P+/+ MEFs (19.6 h and 31.6 h, respectively, in Fig. 4C). Notably, a similar discrepancy was measured also for the doubling time of steady-state levels of total proteins [20.8 h and 29 h, respectively, in Fig. 3C] and nucleic acids [20.5 and 28.7, respectively, in Fig. 5F].

To determine what part of the cell cycle is shortened in rpS6P−/− MEFs, we performed flow cytometry on asynchronous MEF cultures, using DNA content as a sorting parameter. The percentage of rpS6P−/− MEFs in G1 phase decreased from 53.5 in rpS6P+/+ MEFs to 41.6 in rpS6P−/− MEFs [Fig. 4D]. This change reflects a shortening of the G1 phase from 16.4 h [percent in G1 × tP] in wild-type MEFs to 8.2 h in rpS6P−/− MEFs, which can account for most of the apparent difference in the doubling times between these cell types. Finally, if the faster division rate of cells in rpS6P−/− embryos compensates for their smaller size, then the number of cells in rpS6P−/− newborns might exceed that of wild-type newborns. Indeed, Figure 4E shows that the DNA content of the former was 15% higher than that of the latter [3.074 ± 0.064 (n = 4) and 2.681 ± 0.11 (n = 4)] micrograms of DNA per gram of body weight, respectively.

The small-size phenotype of rpS6P−/− MEFs can result from an accelerated cell cycle in the face of an unchanged rate of cell growth. Under such circumstances, blocking cell division in the presence of nutrients should eliminate the size difference between rpS6P−/− and rpS6P+/+ MEFs. This difference will remain, however, if rpS6P−/− MEFs are defective in their growth capacity. In order to distinguish between these two possibilities, we disconnected cell growth from cell cycle progression by 24 h of treatment with 30 µM aphidicolin [DNA polymerase-α inhibitor], which completely arrested cell division [Fig. 5A]. This treatment elicited a small increase in the size of rpS6P−/− and rpS6P+/+ MEFs (9% and 6%, respectively), yet the latter remained 21% smaller than the former [381 ± 9 and 483 ± 3 FSC-H units, respectively, in Fig. 5B]. To further examine this issue, we monitored the cell size and cell doubling time of immortalized rpS6P+/+ and rpS6P−/− MEFs. Figure 5C shows that the size of immortalized rpS6P+/+ MEFs increased to the extent that it equaled with that of rpS6P−/− MEFs (456 ± 6 and
The smaller size of rpS6<sup>−/−</sup> MEFs is not a by-product of their faster cell division. (A) MEFs (passage 3) were seeded in a 96-well plate at a density of 2 x 10<sup>4</sup> per well. On the following day, cells were treated for 24 h with increasing concentrations of aphidicolin, and <sup>3</sup>H-thymidine incorporation during the last 3 h was measured as described in Materials and Methods. Each point represents an average size of four wells. (B) rpS6<sup>−/−</sup> and rpS6<sup>+/−</sup> MEFs (passage 3) were either untreated (Control) or treated with 30 μM aphidicolin for 24 h, and their size was determined as described in Materials and Methods. The average size is presented as the mean FSC-H ± SEM (n = 4). [*] P < 0.01 versus untreated cells. (C) The size of nonsynchronous immortalized rpS6<sup>−/−</sup> [black curve] and rpS6<sup>+/−</sup> MEFs [gray curve] was determined as described in Materials and Methods. The average size is presented as the mean FSC-H ± SEM of the number of cultured plates in parentheses. (D) Immortalized MEFs were seeded in 96-well plates at a density of 4 x 10<sup>4</sup> per well. Proliferation was monitored by measuring the A<sub>490</sub> of the methylene-blue dye extracted from stained cells [Oliver et al. 1989]. Absorbance measured 6 h after plating was set arbitrarily at 1, and absorbance measured at later time points (average ± SEM, n = 12 for each time point) was normalized to that value. (E) rpS6<sup>−/−</sup> and rpS6<sup>+/−</sup> MEFs (passage 3) were either untreated (Control) or treated with 20 nM rapamycin for 48 h, and their size was determined as described in Materials and Methods. The average size is presented as a mean FSC-H ± SEM (n = 3). [*] P < 0.05 versus untreated cells. (F) rpS6<sup>−/−</sup> and rpS6<sup>+/−</sup> MEFs (passage 3) were either untreated or treated with 20 nM rapamycin for 48 h, and the rate of proliferation was measured as described in D. Each time point is an average ± SEM (n = 6 to 12). The bars representing the SEM in D and F are smaller than the symbols’ size.

462 ± 12 FSC-H units, respectively). However, this increase in size was not accompanied by lengthening of the t<sub>d</sub> [Fig. 5D], as would be expected if the size was inversely proportional to the division rate. Likewise, immortalization led to a twofold shortening of the doubling time of wild-type MEFs [from 30.6 h before [Fig. 4C] to 15.1 h after [Fig. 5D] immortalization]. Yet, despite this acceleration in their proliferation rate, their size did not decrease [433 and 462 FSC-H units before [Fig. 4A] and after [Fig. 5C] immortalization, respectively]. Taken together, these results lend support to the notion that the small-size phenotype of primary rpS6<sup>−/−</sup> MEFs reflects impaired growth, rather than being a by-product of accelerated cell division.

**Rapamycin failed to decrease the size of rpS6<sup>−/−</sup> MEFs**

Previous reports have demonstrated that treatment of mammalian cells by rapamycin, an mTOR inhibitor, decreases their size. This mTOR-dependent regulation of the cell size involves its downstream targets, S6K1 and 4E-BP [Fingar et al. 2002; Ohanna et al. 2005]. Here we show that the size of rpS6<sup>−/−</sup> MEFs decreased by 9% upon rapamycin treatment, whereas the size of rpS6<sup>+/−</sup> MEFs was unaffected by this treatment [Fig. 5E]. It should be noted, however, that the apparent rapamycin resistance of rpS6<sup>−/−</sup> MEFs is confined to the effect of this drug on the cell size, as their proliferation rate was significantly reduced by rapamycin treatment, albeit to a lesser extent than that of wild-type MEFs (Fig. 5F).

**Glucose homeostasis is compromised in rpS6<sup>−/−</sup> mice**

It has previously been shown that insulin secretion closely correlates with the size of β-cells [Giordano et al. 1993; Pende et al. 2000]. Mice deficient for S6K1 exhibited impaired glucose homeostasis, due to insufficient insulin secretion in response to glucose load. The reason for this defect was proposed to be the small size of β-cells in S6K1<sup>−/−</sup> mice [Pende et al. 2000]. Neither rpS6 nor any of the other potential S6K1 substrates has been implicated in this defect. To directly address this issue, we first set out to examine whether the phosphorylatable serine residues in rpS6 play a role in size regulation of β-cells. To this end, pancreatic sections were subjected to immunostaining and morphometry. DAPI staining of nuclei in such sections demonstrated a greater density of β-cells than in wild-type islets [Fig. 6A,B]. Accordingly, the higher density is reflected in an apparent smaller cytoplasmic volume of β-cells, as exemplified by insulin immunostaining of the same islets [Fig. 6C,D]. Measuring the density of β-cells in a constant area within multiple islets indicated that insulin-positive cells were more densely packed in rpS6<sup>−/−</sup> islets [Fig. 6E]. This difference implies that the average section area and volume of an rpS6<sup>−/−</sup> β-cell are 25% and 35%, respectively, smaller than those of the wild-type counterpart. Similar results were obtained when the total number of β-cells, in 10 islets from each genotype, was normalized to the entire area occupied by these cells in each islet (data not shown). Evidently, the reduced size of β-cells is specific, as the density of rpS6<sup>−/−</sup> acinar cells, which comprise most of the pancreatic mass, is similar to that of the wild-type cells [Fig. 6F]. Likewise, a similar size of T lymphocytes, derived
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from lymph nodes of adult rpS6P−/− or rpS6P+/− mice and activated with anti-CD3 and anti-CD28, was measured by flow cytometry [data not shown]. It appears, therefore, that normal growth of β-cells is more reliant on the phosphorylatable serine residues than growth of other examined cell types in the adult mouse.

Next, we asked whether the impaired glucose homeostasis, which was described for S6K1 knockout mice (Pende et al. 2000), could also be attributed to a defect in rpS6 phosphorylation. Figure 7A shows that the blood glucose level in fasted rpS6P−/− mice [77 ± 4 mg/dL, n = 14] was quite similar to that of rpS6P+/− mice [75 ± 5 mg/dL, n = 11]. However, the mutant mice exhibited significant reduction in their glucose disposal capacity, as exemplified by the higher and prolonged hyperglycemic response following a glucose challenge [Fig. 7A]. This impairment might reflect reduced glucose uptake by peripheral tissues and/or pancreatic failure. Figure 7B shows that in the fasting state, rpS6P−/− mice had significantly lower serum insulin concentrations than the wild-type mice [rpS6P+/− mice 388 ± 51 pg/mL; rpS6P−/− mice, 171 ± 22 pg/mL; P < 0.01]. Measurement of pancreatic insulin of age-matched female rpS6P+/+ and rpS6P−/− mice revealed a twofold decrease in total insulin content in the pancreas of knock-in mice relative to that of wild-type mice [Fig. 7C]. An intriguing question is whether the lower content of pancreatic insulin reflects reduced total β-cell mass. To draw a quantitative assessment of this latter parameter, we subjected multiple sections of each pancreas to immunostaining and morphometry. The results presented in Figure 7D clearly show that the total β-cell mass is similar in the pancreas of both genotypes. Moreover, this similarity implies an increase by a factor of ~1.5 in the number of β-cells in rpS6P−/− mice in order to compensate for their smaller size. Hence, the overall reduction in insulin content by a factor of 2 seems to reflect a decrease by about threefold in the rate of insulin accumulation per cell.

Finally, to determine whether the hypoinsulinemia is accompanied by alterations in insulin resistance, we carried out insulin tolerance tests. Figure 7E demonstrates that not only did the peripheral tissues in the knock-in

Figure 6. Pancreatic β-cells are selectively smaller in rpS6P−/− mice. Pancreatic sections from rpS6P−/− and rpS6P+/+ mice were stained for DNA by DAPI (blue in A, B, red in C, D) and for insulin (green in C, D). Panels C and D represent insertion of the RGB image from the DAPI staining into the red channel of the respective image of the insulin staining. Shown are representative islets. Bar, 50 µm. The density of β-cells (E) and acinar cells (F) was assessed by counting the number of nuclei in multiple 2500-µm² squares within islets that contain only insulin-positive cells or within exocrine pancreas that contains only acinar cells, respectively. Values are presented as a mean ± SEM of ~100 determinations done with five to seven islets for each cell type in four female mice of each genotype. (** P < 0.001 versus rpS6P+/+ mice.

Figure 7. Glucose homeostasis is damaged in rpS6P−/− mice. (A) Glucose tolerance test. Blood glucose concentrations before and after intraperitoneal injection of 2.5 g of D-glucose per kilogram of body weight in 6-wk-old mice fasted for 17 h. The data represent an average ± SEM for 11 rpS6P−/− mice (four males and seven females) and 14 rpS6P+/+ mice [nine males and five females], respectively. (*) P < 0.05; (** P < 0.005 versus rpS6P+/+ mice. (B) Insulin concentrations in 2-mo-old female mice fasted for 16 h were determined by tail bleeding. Values depict an average ± SEM for seven animals each. (* P < 0.005 versus rpS6P+/−/− mice. (C) Total pancreatic insulin content. Pancreases from age-matched female mice were removed, and the insulin content was measured as described in Materials and Methods. The data represent an average ± SEM for five animals each. (*) P < 0.05 versus rpS6P+/−/− mice. (D) Total β-cell mass. Pancreases from four rpS6P+/−/− and seven rpS6P−/−/− age-matched female mice were removed, and the total β-cell mass was determined as described in Materials and Methods. The data are presented as a mean ± SEM. (E) Insulin tolerance test. Blood glucose concentrations before and after intraperitoneal injection of 0.25 U of insulin/kilogram of body weight in 2-mo-old fed female mice. The data represent an average ± SEM for five animals each. (*) P < 0.05; [**] P < 0.005 versus rpS6P+/−/− mice.
mice not display insulin-resistance, they actually exhibited greater sensitivity, as exemplified by the greater glucose-lowering effect.

It appears, therefore, that the diminished glucose disposal capacity of rpS6<sup>−/−</sup> mice primarily reflects relative insulin deficiency, rather than insulin resistance in peripheral tissues. Taken together, our results clearly show that the phosphorylatable serine residues in rpS6 are critical for the normal size and function of β-cells, as well as the insulin sensitivity in peripheral tissues. Moreover, these observations imply that rpS6 is the prime substrate through which S6K1 exerts its effect on glucose homeostasis.

**Discussion**

The first attempt to elucidate the role of rpS6 phosphorylation was carried out in yeast, whose rpS6, like its mammalian counterpart, is phosphorylated upon mitogenic stimulation, yet at only two serine residues. Serine-to-alanine substitution of these phosphorylatable residues had no detectable effect on yeast growth under a wide variety of nutritional conditions (Johnson and Warner 1987). In light of this observation, the physiological role of yeast rpS6 phosphorylation has so far remained obscure. Interestingly, conditional knockout of both alleles of rpS6 in adult mouse liver has demonstrated the requirement for this protein for ribosome assembly and growth and not from accelerated cell cycle progression (Schumacher et al. 1999; Conlon et al. 2004). Interestingly, transgenic mice, which overexpress active Akt1 specifically in their myoblasts and rpS6<sup>−/−</sup>−/− MEFs is reminiscent of that exhibited by S6K1<sup>−/−</sup> myoblasts (Ohanna et al. 2005). It appears, therefore, that rpS6 phosphorylation is a critical effector of mTOR in regulation of cell growth. Notably, the small size of S6K1<sup>−/−</sup>−/− myoblasts is apparent, even though their rpS6 is still phosphorylated, most probably by S6K2 (Ohanna et al. 2005). Conceivably, once the growth of a specific cell lineage is blocked by a temporary deficiency of rpS6 phosphorylation, as a result of S6K1 deficiency, the small size is maintained thereafter, regardless of a later phosphorylation of rpS6 by a different kinase.

The smaller size of β-cells in rpS6<sup>−/−</sup>−/− islets might also result from selective decreased cell growth. However, in the absence of data regarding the rate of division of these cells, we cannot exclude the possibility that their smaller size is secondary to an accelerated division. It appears, however, that the small cell phenotype is confined to only a subset of cells. Thus, either acinar cells in the pancreas (Fig. 6) or activated T lymphocytes from lymph nodes (data not shown) displayed a similar size regardless of the rpS6 genotype. Interestingly, the similar total β-cell mass in both genotypes, despite the smaller size of individual β-cells in rpS6<sup>−/−</sup>−/− islets, suggests that this tissue operates a mass-control mechanism rather than monitoring cell number. Likewise, the similar birth weight of rpS6<sup>−/−</sup>−/− mice (Fig. 4B), despite a smaller size of embryonic cells from these mice (Fig. 4A), suggests that a similar mechanism functions also at the organismal level.

Mammalian rpS6 is phosphorylated in response to multiple external signals, which are transduced through the phosphatidylinositol 3-kinase (PI3-kinase)/mTOR pathways (for review, see Fingar and Blenis 2004; Hay and Sonenberg 2004). Genetic and biochemical studies have demonstrated that all constituents of these two pathways, examined thus far are involved in the size regulation of mammalian cells (for review, see Fingar and Blenis 2004). Interestingly, transgenic mice, which overexpress active Akt1 specifically in their β-cells, display an increase in both the number and size of their β-cells (Bernal-Mizrachi et al. 2001; Tuttle et al. 2001). It should be noted that constitutive activation of Akt1 by its targeting to the membrane, as conducted in both these studies, leads to constitutive activation of S6K1 (Burginger and Coffer 1995). The apparent opposite effects on β-cell size of constitutively active Akt1, on the one hand, and of deficiency of S6K1 or rpS6 phosphorylation (Pende et al. 2000; this study), on the other hand, suggest that Akt1 induces enlargement of β-cells, at least partly, by activation of rpS6 phosphorylation.

**rpS6 phosphorylation and glucose homeostasis**

We have observed a twofold reduction in circulating insulin levels in rpS6<sup>−/−</sup>−/− mice in comparison to wild-type mice (Fig. 7B). This decrease is similar to that reported for S6K<sup>−/−</sup>−/− mice (Pende et al. 2000), and might reflect diminished accumulation in and/or secretion from β-cells. Indeed, β-cell size is known to have a pro-
nounced effect on insulin secretion, such that a 35% reduction in cell size, as reported here, can lead to a 40%–50% reduction in insulin secretion (Giordano et al. 1993). However, even though the relative radius of these cells, as calculated from their relative density [Fig. 6E], is 25% smaller than that of the wild-type cells, the 1.5-fold higher number of the former renders their total surface area a nonlimiting parameter. It appears, therefore, that the decreased pancreatic insulin content, and possibly an impaired mechanism of insulin secretion, accounts for the apparent lesion in glucose homeostasis.

Interestingly, the apparent glucose intolerance in rpS6P−/− mice is reminiscent of the impaired glucose tolerance observed in offspring of rats that were undernourished during pregnancy, or in adult human beings after prenatal exposure to famine (Ravelli et al. 1998 and references therein). Moreover, a low-protein diet during pregnancy leads to reduction of pancreatic cell proliferation, islet size, islet vascularity, and insulin content in the fetal rat [Dahri et al. 1991]. Possibly, malnutrition during pregnancy leads to insufficient signals through mTOR, an integrator of nutritional signals (Fingar and Blenis 2004; Proud 2004), which in turn leads to hypophosphorylation of rpS6 during a critical stage of pancreatic development and consequently to impaired pancreatic function in the adult organism. It should be pointed out, however, that the effect of perinatal famine on the size of β-cells, a hallmark of rpS6P−/− and S6K1−/− mice, is currently unknown. Consequently, assessment of the parallelism between these mutants and the starved embryos should wait till this information is available.

In addition to the pancreatic phenotype, rpS6P−/− mice display increased insulin sensitivity in peripheral tissues (Fig. 7E), which is reminiscent of that observed in S6K1-deficient mice (Pende et al. 2000). It has recently been proposed that S6K1 exerts a negative feedback loop on insulin receptor substrate 1 (IRS1) by phosphorylation of a serine residue, and thus blocks its signal transduction activity [Harrington et al. 2004; Um et al. 2004]. However, if increased insulin sensitivity in S6K1−/− mice, indeed, results from the elimination of serine phosphorylation, it is quite puzzling how the lack of phosphorylatable serine residues in rpS6 is able to phenocopy the effect of S6K1 deficiency. One plausible explanation is that unphosphorylatable rpS6 is associated with an increased activity of an IRS1 serine phosphatase.

Notably, rpS6 in our knock-in mice not only cannot be phosphorylated but also contains five serine-to-alanine substitutions. Hence, it can be argued that the observed phenotype results from impeded ribosomal function due to these structural modifications, rather than being phosphorylation-free. Nonetheless, many of the phenotypic manifestations of rpS6P−/− knock-in mice are similar to those observed in S6K1 knockout mice. These include smaller-size β-cells, diminished insulin content in pancreas, hypoinsulinemia, glucose intolerance, and insulin hypersensitivity in peripheral tissues (Pende et al. 2000, this study). This apparent similarity suggests that it is the failure to phosphorylate rpS6 that can account for the common defects in both types of mutants. Accordingly, our results strongly imply that rpS6 is the missing effector of S6K1 in regulation of the size and function of β-cells. It should be stressed, however, that S6K1−/− mice, unlike rpS6P−/− mice, also display an in utero developmental defect manifested in smaller birth size (Shima et al. 1998), and the disruption of both S6K1 and S6K2 leads to decreased viability due to perinatal lethality [Pende et al. 2004]. Clearly, these phenotypes indicate that S6K1 and/or S6K2 exert their distinct effects via targets other than rpS6. Indeed, S6K has been implicated in an increasing list of cellular processes through a wide variety of newly established substrates (de Groot et al. 1994; Wilson et al. 2000; Harada et al. 2001; Wang et al. 2001; Harrington et al. 2004; Raught et al. 2004, Richardson et al. 2004).

**rpS6 phosphorylation and protein synthesis**

Our present results have established that rpS6 phosphorylation is dispensable for efficient translation of TOP mRNAs. This observation, together with the report that TOP mRNAs are efficiently translated in S6K1−/−/S6K2−/− cells [Pende et al. 2004], has unambiguously demonstrated that translational activation of TOP mRNAs relies on neither S6K activity nor on rpS6 phosphorylation. It should be mentioned that rapamycin ubiquitous blocks the activation of S6K1 and S6K2 and rpS6 phosphorylation (Chung et al. 1992; Shima et al. 1998), and suppresses translational activation of TOP mRNAs in some cell lines (Stolovich et al. 2002 and references therein). However, the fact that TOP mRNAs are translationally controlled in an S6K- and rpS6-phosphorylation-independent fashion [Pende et al. 2004; this study] implies that mTOR or another, as yet unknown, rapamycin-sensitive target exerts its regulatory role on TOP mRNAs in an S6K1- and S6K2-independent fashion.

The data presented here have shown that rpS6 phosphorylation is dispensable for efficient polysomal association of liver ribosomes [Fig. 3A]. Moreover, the rates of global protein synthesis and accumulation are even increased in rpS6P−/− MEFs [Fig. 3B,C]. This latter observation implies that protein synthesis, at least in this cell type, is down-regulated by rpS6 phosphorylation. Nevertheless, one plausible explanation for the discrepancy between the enhancements in the rates of protein synthesis and protein accumulation (2.5-fold and 1.4-fold higher, respectively, in rpS6P−/− MEFs) is an increased protein degradation in the mutant cells, which partially counterbalances the higher synthesis rate. However, validation of this hypothesis should await further studies. Furthermore, the enhanced protein synthesis in rpS6P−/− MEFs cannot be attributed to the slight increase in the rate of translation elongation, as measured in these cells [Fig. 3D]. Likewise, it cannot be ascribed to activation of S6K kinase and/or inactivation of elf-4E-binding protein 1 (4E-BP1), as their phosphorylation in rpS6P−/− MEFs was not higher than that detected in rpS6P−/− MEFs [data not shown]. Nonetheless, the tissue-specific deleterious effect in the pancreas of glucose-
loaded rpS6<sup>P−/−</sup> mice suggests that rpS6 phosphorylation is required for the synthesis or function of some critical proteins necessary for coping with hyperglycemia.

Conceivably, the phosphorylation of rpS6 increases the affinity of the 40S ribosomal subunit to a subclass of mRNAs, and thus promotes their efficient translation. However, we cannot rule out the possibility that the phosphorylated rpS6 does not affect protein synthesis, but instead serves as a docking site for a protein(s), which consequently becomes active or inactive, and thus affects the cell physiology. It should be mentioned that rpS6 is not the only ribosomal protein that undergoes regulated phosphorylation. It has recently been shown that interferon-γ-dependent phosphorylation of rpL13a leads to its release from the 60S ribosomal subunit and subsequently to its binding to eukaryosm RNA, and consequently to its silencing (Mazumder et al. 2003).

Materials and methods

Generation of rpS6<sup>P−/−</sup> mice

A 6.85-kb DNA fragment, containing mouse rpS6 gene as well as 0.9 and 3.3 kb of 5′- and 3′-flanking sequences, respectively, was assembled from two partially overlapping genomic clones, isolated [Pata and Metspalu 1996] from a 129Sv/J library [Stratagene]. A targeting vector was constructed by inclusion of the following modifications: [1] substitution of 6 nucleotides [nt] within the coding sequence to yield replacement of all five phosphorylatable serine residues by alanine residues; [2] insertion of a diphtheria toxin A chain gene driven by the PGK promoter (a negative selectable marker), immediately upstream of the rpS6 genomic sequence to enable enrichment of correctly targeted clones (Fig. 1A). The targeting vector was linearized by NotI digestion and electroporated into ES cells [35 × 10<sup>4</sup>] from the R1 (129Sv × 129Sv-CP) strain. The cells were grown in the presence of G418, and live correctly targeted clones were identified by Southern blot hybridization of genomic DNA, with two different probes (Fig. 1A). Three correctly targeted clones were aggregated with morulae-stage embryos to produce chimeras. Male chimeras were mated with ICR females to produce heterozygous RP6<sup>P+/−</sup> and RP6<sup>P−/−</sup> male mice (6–13 wk old) under xylazine (10 µg/g body weight) and ketamine (450 µg/g body weight) anesthesia.

Partial hepatectomy

Partial hepatectomy resulting in the removal of the median and left lateral lobes of the liver was performed on age-matched RP6<sup>P+/+</sup> and RP6<sup>P−/−</sup> male mice [6–13 wk old] under xylazine (10 µg/g body weight) and ketamine (450 µg/g body weight) anesthesia.

Determination of rpS6 phosphorylation

Primary MEFs were serum starved for 48 h in 60-mm plates, and then the medium was replaced with 0.5 mL of phosphate-free Dulbecco’s Modified Eagle’s Medium containing 10% dialyzed fetal bovine serum. After 30 min of preincubation at 37°C, cells were labeled with 20 µL (200 µCi) of [3H]thymidine (8000 Ci/mmol, Amer sham) for 4 h. Cells were pelleted at 2000 rpm in a microtuge at 4°C, resuspended in 150 µL of resuspension buffer [10 mM NaCl, 10 mM Tris-HCl at pH 7.4, 15 mM MgCl<sub>2</sub>, 50 mM β-glycerophosphate, 20 mM PMFS, 10 µg/mL aprotinin, and 10 µg/mL leupeptin] and lysed by adding 20 µL of lysis buffer (10% Triton X-100, 10% deoxycholate). The cell lysate was centrifuged at 4°C for 2.5 min at 14,000 rpm. One-hundred-fifty microliters of the supernatant were diluted with 350 µL of polysomal buffer [25 mM Tris-HCl at pH 7.4, 10 mM MgCl<sub>2</sub>, 25 mM NaCl, 0.05% Triton X-100, 0.14 M sucrose, 500 µg/mL heparin, 10 µg/mL aprotinin, and 10 µg/mL leupeptin]. Polysomes were spun down at 55,000 rpm for 20 min at 4°C in a Beckman TLA55 rotor, and the polysomal pellet was dissolved in buffer H. Proteins were size-fractionated by SDS-PAGE, and phosphoproteins were identified by autoradiography.

Polysomal fractionation and RNA analysis

Polysomal fractionation and RNA analysis were performed as previously described [Tang et al. 2001]. Preparation of liver cytoplasmic extracts was carried out as described [Aloni et al. 1992]. The molecular probes used in blot hybridizations were previously described [Aloni et al. 1992].

Rate of protein synthesis

Subconfluent cultures of MEFs (passage 2) from RP6<sup>P+/+</sup> and RP6<sup>P−/−</sup> mice were grown in six-well plates. Cells were washed...
with PBS and then replenished with DMEM without methio-
nine and cysteine [Invitrogen], containing 10% dialyzed fetal calf serum [Beit Haemek], 2 mM glutamine, 100 units/mL penicil-
lin, and 0.1 mg/mL streptomycin. One hour later, cells were
labeled with 10 µCi of EXPRE35S35S [PerkinElmer] per well for 30
or 120 min and were then washed once with PBS containing
methionine and cysteine, 10 mM each. Cells were lysed in 75 µL
of buffer H without aprotinin and leupeptin. Aliquots of 10 µL
were taken for precipitation in 10% trichloroacetic acid (TCA)
containing methionine and cysteine, 10 mM each. The precipi-
tates were collected on filter (GF/C) and washed thrice with 5%
TCA and once with ethanol. Filters were dried down and sub-
jected to liquid scintillation counting using Opti-Fluor [Packard
Biosciences]. Similar aliquots were used for protein determina-
tion [Lowry et al. 1951]. The protein synthesis at each time
point was calculated as TCA-precipitable counts per minute
divided by micrograms of protein in the same sample.

Measurement of ribosome half-transit time

Cells (³ 10⁷) were suspended in 5 mL of labeling medium
[Basal Medium-Eagle [Biological Industries] supplemented with
10% dialyzed bovine calf serum, 3.5 g glucose/L, and 2 mM
glutamine] for 20 min and then 10 µCi/mL [4,5-³H]leucine
[Amersham Biosciences] was added. At the times indicated,
cells were harvested, pelleted, and resuspended in 0.5 mL of RSB
(10 mM NaCl, 10 mM Tris-HCl at pH 7.4, 15 mM MgCl₂, and
100 µg/mL heparin). Cells were lysed by adding 70 µL of Lysis
buffer (10% Triton X-100, 10% deoxycholate) and subjecting
them to 3 sec on Vortex mix before and after 3 min of incuba-
tion on ice. Nuclei and mitochondria were pelleted by centrifug-
ation at 10 min on maximum speed in a microfuge at 4°C.

Five-hundred microliters of the PMS were mixed with an equal
volume of Polysomal buffer (25 mM Tris-Cl at pH 7.4, 10 mM
MgCl₂, 25 mM NaCl, 0.05% Triton X-100, 0.14 M sucrose, 500
µg/mL heparin), and 450 µL was removed to measure incorpo-
ration of [³H]leucine into total protein [nascent and completed].
Polysomes were pelleted by centrifugation of the remaining su-
pernatant at 55,000 × g for 20 min at 4°C in a Beckman TL15S
rotor. Four-hundred-fifty microliters of PRS were removed to
measure the incorporation of [³H]leucine into completed pro-
tein. PMS and PRS samples were mixed with equal volumes of
20% TCA containing 10 mM leucine, placed on ice for 20 min,
and then TCA precipitates were collected on a glass fiber filter
(GF/C, Whatman). Filters were washed with ice-cold 10% TCA,
rinsed once with ethanol, and air-dried before being subjected to
liquid scintillation counting.

Immunofluorescence microscopy of pancreas sections

Five-micrometer-thick sections from formalin-fixed, paraffin-
embedded pancreases were prepared. Slides were rehydrated,
blocked with 1% BSA and 10% goat serum in phosphate buff-
cered saline, 0.9% NaCl, and 0.3% Triton X-100 [pH 7.4] (PBST),
and incubated overnight at 4°C with primary antibodies. After
washing in PBST, slides were incubated for 1 h with fluorescent
secondary antibodies, washed again, incubated briefly with 4',6-
diamidino-2-phenylindole (DAPI), and mounted. Digital images
of islets and acinar tissue were obtained using an Olympus
BX51 microscope (200× magnification), based on staining for
insulin and amylase, respectively. The number of nuclei in a con-
stant area (2500 µm²) was counted manually using Image-Pro
Plus software [Media Cybernetics]. The primary antibodies
used were guinea pig anti-insulin [DAKO] and rabbit anti-amy-
lase [Sigma]. The secondary antibodies used were CY2-anti-
guinea pig [Jackson] and CY3-anti rabbit [Jackson]. All antibod-
ies were used at 1:200 dilution.

Glucose and insulin tolerance tests and measurements of
insulin levels

A glucose tolerance test was performed on fasted (17 h) mice by
injecting D-glucose intraperitoneally at a dose of 2.5 g/kg of
body weight. Whole venous blood was obtained from the tail at
the indicated time points after the glucose load. Blood glucose
levels were measured using a glucometer (Bayer). An insulin
tolerance test was performed on fed mice by injecting human
insulin [Humalog; Eli Lilly Corp.] intraperitoneally at a dose of
0.25 U/kg of body weight. To measure insulin concentration,
blood was collected from the tail of 16-h-fasted mice and cen-
trifuged. Insulin levels were measured in 5 µL of plasma using
an enzyme-linked immunosorbent assay (ELISA) kit for mouse
insulin [Merckodia AB]. Measurements of pancreatic insulin
were carried out with pancreas homogenates using a rat insulin
radioimmunoassay kit [Linco]. A t-test was used for statistical
analysis, and a P value of 0.05 or less was considered significant.

Measurement of total β-cell mass

The whole pancreas was removed and weighed, fixed in zinc
formalin for 4 h, and embedded in paraffin. Five-micrometer-
thick sections were prepared through the whole block, and two
consecutive slides every 100 microns were stained: one with
guinea pig anti-insulin antibodies followed by HRP-conjugated
anti-guinea pig antibody, and the other with hematoxylin and
cosin. Stained slides were scanned at 4800 × 4800 dpi using a
standard desktop scanner, and total tissue area, as well as total
insulin-positive area, was calculated using Image-Pro Plus soft-
ware. The fractional area occupied by β-cells was multiplied by
pancreas weight to determine total β-cell mass. Control exper-
iments using high-resolution images confirmed that this method
takes into account all β-cells including most single cells.

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