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ORIGINAL ARTICLE

Evidence for cell cycle-dependent, rapamycin-resistant phosphorylation of ribosomal protein S6 at S240/244

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Abstract The ribosomal protein S6 is essential for the formation of the subunits of higher eukaryotic ribosomes, and S6 heterozygosity leads to early embryonal lethality in mice. S6 is phosphorylated at clustered residues S235/236 and S240/244 upon numerous physiological and pathological stimuli. So far, the S6Kinases, S6K1 and S6K2 are the only proven S6 S240/244 phosphorylating enzymes in mammalian cells. The activity of these S6Kinases is strictly regulated via the mammalian target of rapamycin (mTOR) enzyme complex with raptor, named mTORC1. In time course experiments with the mTORC1 inhibitor rapamycin we here demonstrate rapamycin-resistant phosphorylation of the ribosomal protein S6 at S240/244. Serum-restimulation experiments further demonstrated that this rapamycin-resistant S6 240/244 phosphorylation is induced via serum factors in a cell cycle-dependent manner. Our data allow new insights into the regulation of S6 phosphorylation and provide evidence for the existence of rapamycin-resistant S6 phosphorylating kinase activities.

Keywords mTOR · Phosphorylation · Rapamycin · S6 · S6Kinase

Introduction

The serine/threonine kinase Raf links Ras to the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway, which is involved in the regulation of a wide variety of different cellular processes, including tumour development and cell differentiation. The Raf/MEK/ERK cascade leads to ERK phosphorylation and activation of RSK (Shaw and Cantley 2006; Laplante and Sabatini 2009; Fig. 1).

The PI3K/AKT pathway is also centrally involved in growth regulation, proliferation control and cancer cell metabolism. In this cascade, PI3K regulates the activity of the oncogenic kinase Akt (also known as protein kinase B) via PDK1 (phosphoinositide-dependent kinase-1). Akt-mediated phosphorylation at S939 and T1462 downregulates the GTPase activating (GAP) potential of the tumour suppressor protein tuberin, the gene product of the tuberous sclerosis gene 2 (TSC2), toward Rheb (Ras homolog enriched in brain), which is a potent regulator of the mammalian target of rapamycin (mTOR) (Yang and Guan 2007; Dann et al. 2007; Rosner et al. 2004, 2008; Laplante and Sabatini 2009; Fig. 1).

In mammalian cells two structurally and functionally distinct mTOR-containing complexes have been identified. mTORC1 contains raptor (regulatory associated protein of mTOR), mLST8 (also known as GβL) and PRAS40 (proline-rich Akt substrate 40 kDa). mTORC2 consists of the mLST8 protein, rictor (rapamycin-insensitive companion of mTOR) and sin1 (stress-activated protein kinase-interacting protein). Well known substrates of mTORC1 are the S6Ks (ribosomal protein S6 kinases). Especially, phosphorylation of p70S6K1 at T389 is mediated via mTORC1. mTORC2 phosphorylates Akt at S473, what in conjunction with the PDK1-mediated phosphorylation of Akt at T308 drives full activation of Akt (Bhaskar and Hay 2007; Yang and Guan 2007; Dann et al. 2007; Rosner et al. 2004, 2008; Laplante and Sabatini 2009; Fig. 1).

Of all ribosomal proteins, it is ribosomal protein S6 (S6) that has attracted much attention, because it is inducibly phosphorylated. S6 is known to be essential for the

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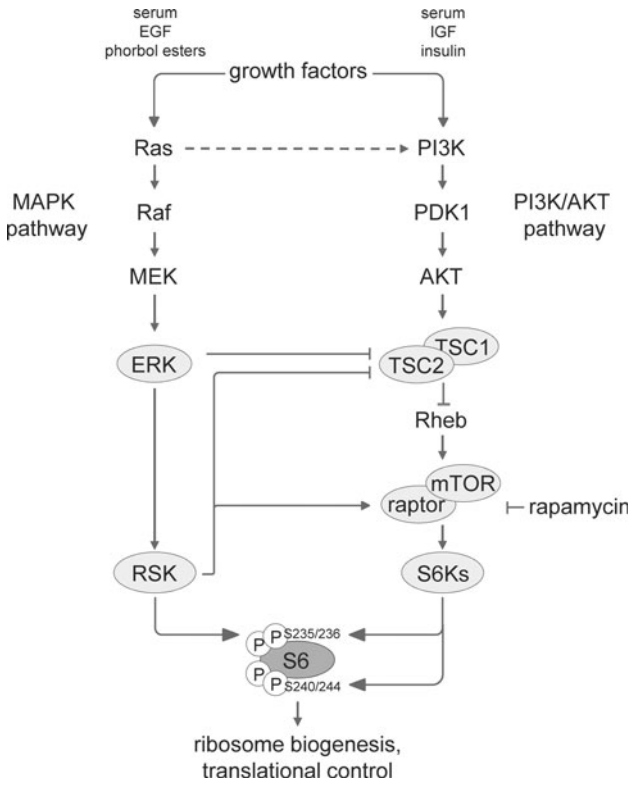

Fig. 1 Site-specific phosphorylation of ribosomal protein S6 (S6) occurs through mTOR-dependent and -independent mechanisms. Schematic presentation of the upstream regulating pathways of S6 phosphorylation (for details see the text)

formation of the subunits of higher eukaryotic ribosomes, and its critical role is highlighted by the finding that S6 heterozygosity leads to early embryonal lethality in mice. In response to various growth-related stimuli S6 is dually phosphorylated at clustered residues S235/236 and S240/244 involving both, the PI3K/AKT and the MAPK signalling pathways. As illustrated in Fig. 1, AKT-mediated phosphorylation of tuberin (TSC2) causes its functional inactivation and leads to enhanced mTORC1 (mTOR/raptor) activity which consequently stimulates S6K-mediated phosphorylation of S6 at S235/236 and at S240/244. In mammalian cells two different genes encode two forms of S6Ks, S6K1 (with its two isoforms p70S6K1 and p85S6K1) and S6K2 (with the isoforms p54S6K2 and p56S6K2). Analyses in mouse cells, deficient in either S6K1 or S6K2, suggest that both are required for full S6 phosphorylation, with the predominance of S6K2. Alternatively, MAPK-activated RSK is known to directly phosphorylate S6 at S235/236 via an mTOR-independent mechanism (Ruvinsky and Meyuhas 2006; Meyuhas 2008).

In addition, the MAPK pathway was demonstrated to impinge on the PI3K/AKT/mTOR pathway at various levels to promote mTOR signalling: Ras binds to and activates the phosphatidylinositol-3-kinase (PI3K). Both, ERK- and RSK-mediated, inactivating phosphorylation of tuberin and RSK-mediated, activating phosphorylation of the mTORC1 component raptor enhance mTOR activity and downstream signalling providing evidence for an mTOR-dependent, rapamycin-sensitive mechanism of S6 phosphorylation via the MAPK pathway. Still, in accordance with this model, mTOR-regulated S6Ks remain the only S6 S240/244 phosphorylating kinases identified so far (Bhaskar and Hay 2007; Yang and Guan 2007; Dann et al. 2007; Rosner et al. 2004, 2008; Laplante and Sabatini 2009; Fig. 1).

Rapamycin is a macrolide antibiotic, made by *Streptomyces hygroscopicus*. Rapamycin bound to the protein FKBP12 generates a drug-receptor complex that binds and inhibits mTORC1. FKBP12-rapamycin suppresses the assembly of mTOR/raptor. Since it does not bind to preformed mTORC2, rapamycin was originally thought to only inhibit mTORC1. However, recently it was shown that long-term treatment also suppresses the function of mTORC2 (Sarbassov et al. 2006; Rosner et al. 2008).

In this study we made use of rapamycin to block mTOR activity in primary non-transformed non-immortalized human cells. However, rapamycin time course experiments revealed that there exists a rapamycin-resistant phosphorylation of the ribosomal protein S6 at S240/244. Serum-restimulation experiments further demonstrated that this rapamycin-resistant S6 240/244 phosphorylation is induced via serum factors in a cell cycle-dependent manner.

Materials and methods

Cell culture

IMR-90 cells are foetal lung-derived, non-transformed, non-immortalized human diploid fibroblasts with finite lifetime and were obtained from the American Type Culture Collection (ATCC #CCL-186) at passage number 10 (population doubling 25). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) at 4.5 g/l glucose, supplemented with 10% calf serum and antibiotics (30 mg/l penicillin, 50 mg/l streptomycin sulphate) at 37°C and 5% CO_2. To avoid potential effects due to cellular senescence, cells were grown for no more than eight additional passages ($\leq$47 total population doublings) and were regularly analysed by standard karyotyping to confirm a normal diploid karyotype (Rosner and Hengstschläger 2008).

For synchronization in G0/G1 IMR-90 fibroblasts were used at passage number 15 and deprived of serum in medium containing 0.2% FCS for 48 h. Cells were stimulated to re-enter the cell cycle by the addition of 10% serum for another 36 h.

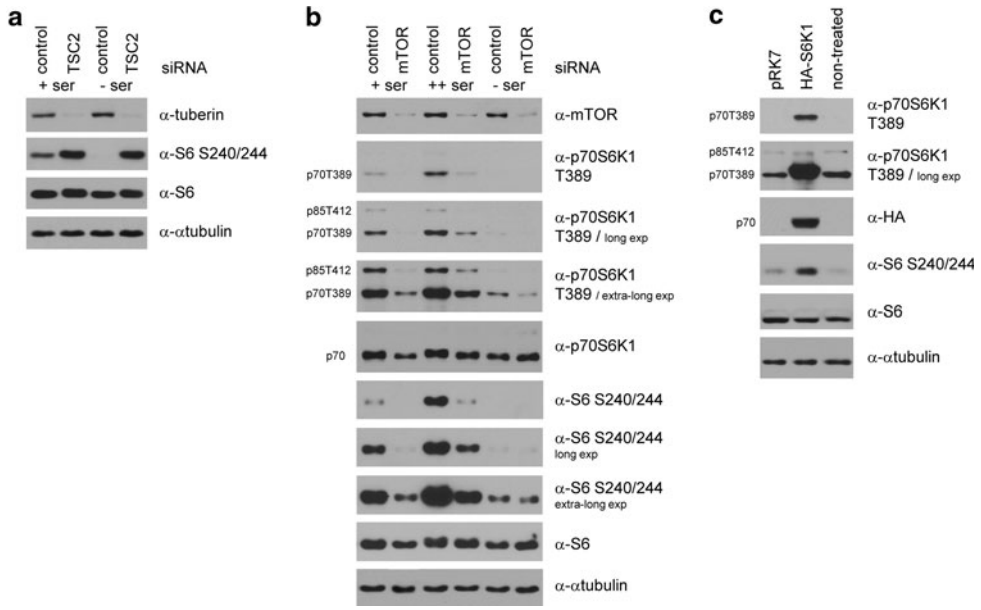

Fig. 2 Modulating the PI3K/AKT/mTOR pathway in primary IMR-90 fibroblasts triggers pronounced effects on S6 phosphorylation at S240/244. **a** IMR-90 fibroblasts were transfected with non-targeting siRNAs or with siRNAs targeting human TSC2 and were grown for a total of 96 h. 16 h before harvest, cells were washed with PBS and starved in serum-free medium (−ser) or left untreated (+ser). So treated cells were lysed and examined for the expression level of tuberin, ribosomal protein S6 phosphorylated at S240/244, total S6 and α-tubulin via immunoblotting. **b** Transfection experiments with mTOR-specific siRNAs were performed as described above except that cells were additionally re-feeded with fresh, serum-containing medium (++ser). Lysates were prepared and analysed for protein expression as indicated. Different western blot exposures of phosphorylated p70S6K1 and S6 are presented to allow better comparison of the corresponding phosphorylation status under different growth conditions (+ser, ++ser, −ser). Here it is important to note that the used α-p70S6K1 and α-p70S6K1 T389 antibodies not only recognize p70S6K1 but also its slower migrating transcript variant p85S6K1 and p85S6K1 phosphorylated at T412, respectively. **c** To study the effects of ectopic p70S6K1 on S6 phosphorylation, IMR-90 cells were transfected with a mammalian expression vector harbouring wild-type, HA-tagged S6K1 or the empty vector control. 48 h after transfection lysates were prepared and immunoblotted for indicated proteins. Cells that were left untreated were analysed in parallel

Experiments involving the mTOR inhibitor rapamycin (Calbiochem) were performed in the absence (DMSO vehicle control) or presence of the drug at final concentration of 100 nM (Rosner et al. 2010).

Transfections

All transfection experiments, including the delivery of siRNA and DNA, were performed using Lipofectamine 2000 transfection reagent (Invitrogen) following the guidelines provided by the manufacturer.

For RNAi-mediated knockdown experiments, pooled siRNAs specifically targeting human TSC2 or mTOR (Dharmacon, ON-TARGET *plus SMART* pool reagents) were delivered to the cells at a final range of 50–100 nM. A pool of four non-targeting siRNAs was used as a control for non-sequence-specific effects. For the ectopic expression of p70S6K1, the following plasmids were used: wild-type rat S6K1, cloned into a mammalian expression vector (pRK7) and N-terminally tagged with HA and the empty vector control. After 48 or 96 h of incubation (for DNA and siRNA transfection experiments, respectively) cells were harvested for analyses (Rosner et al. 2007, 2009).

Protein extraction and immunoblotting

Extracts of cellular total protein were prepared by physical disruption of cell membranes by repeated freeze and thaw cycles. Briefly, cells were washed with PBS and harvested by trypsinization. Pellets were washed twice with ice-cold PBS and lysed in buffer A containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4 supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.3 μg/ml benzamidinchlorid, 10 μg/ml trypsininhibitor by freezing and thawing. Supernatants were collected by centrifugation at 15,000 rpm for 20 min at 4°C and stored at −80°C. Proteins were resolved by 7.5 or 10.5% SDS-PAGE and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the amount of loaded protein. For immunodetection antibodies specific for the following proteins were used: tuberin C-20 (Santa Cruz, #sc-893), mTOR (Cell Signaling, #2972), HA clone 3F10 (Roche, #1 867 423), phospho-p70S6 kinase T389 clone 108D2 (Cell signaling, #9234), p70S6 kinase clone 49D7 (Cell Signaling, #2708), p70S6 kinase (Cell Signaling, #9202), phospho-S6 ribosomal protein S240/244 (Cell Signaling,

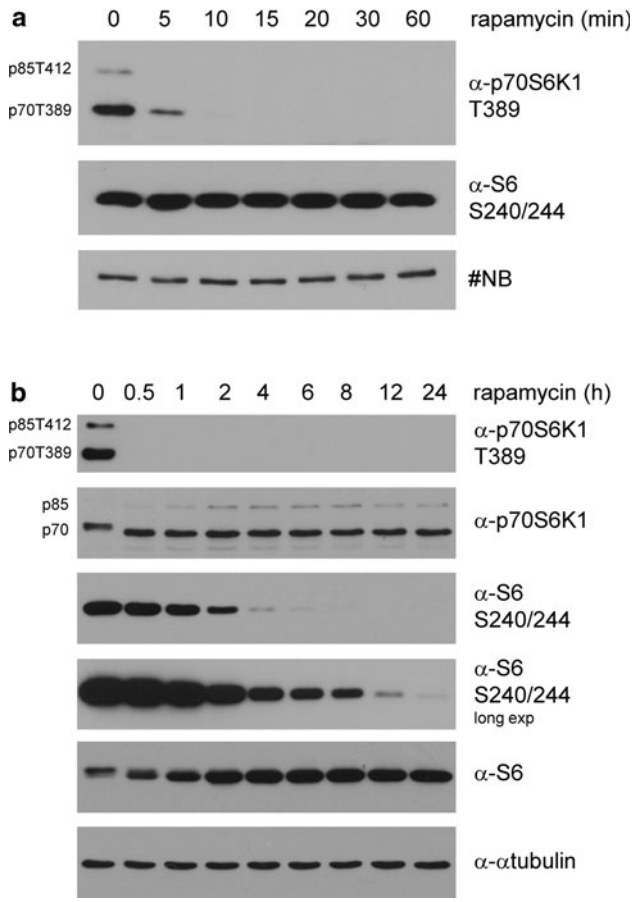

Fig. 3 Rapamycin time course experiments reveal decoupling of phosphorylation of p70S6K1 T389 and of S6 S240/244. **a** Logarithmically growing IMR-90 fibroblasts were treated with 100 nM rapamycin and harvested shortly after its addition at indicated time points. Total lysates were prepared and analysed for the phosphorylated forms of p70S6K1 and S6 as indicated. A non-specific, high-molecular weight band resulting from the detection of T389 phosphorylated p70S6K1 is included to prove equal loading of lysates (#NB). **b** Time course experiments were performed as described above except that different time points ranging from 0.5 to 24 h were analysed. Immunoblotting with antibodies specific for phosphorylated and total forms of p70S6K1 and S6 was performed

#2215), S6 ribosomal protein clone 54D2 (Cell Signaling, #2317) and α-tubulin (DM1A, Calbiochem, #CP06). Rabbit polyclonal or monoclonal antibodies and mouse monoclonal antibodies were detected using anti-rabbit IgG, a HRP-linked heavy and light chain antibody from goat (A120-101P, Bethyl Laboratories) and anti-mouse IgG, a HRP-linked heavy and light chain antibody from goat (A90-116P, Bethyl Laboratories), respectively; the rat monoclonal antibody against HA was detected with goat anti-rat IgG1 (A110-106P, Bethyl Laboratories). Signals were visualized using the enhanced chemiluminescence method (Pierce) (Rosner and Hengstschläger 2007; Burgstaller et al. 2008).

Results and discussion

As already described earlier, the p70S6K1 is a downstream target of mTORC1, which is negatively regulated via tuberin (TSC2). mTORC1 phosphorylates and activates p70S6K1 at T389 to activate the ribosomal protein S6 via phosphorylation at S240/244 (see introduction). In total agreement with this notion, siRNA-mediated downregulation of endogenous tuberin triggered a significant increase in S6 phosphorylated at residues S240/244 (Fig. 2a). Knock-down of endogenous mTOR via specific siRNA treatment caused a decrease of intracellular phosphorylation of p70S6K1 at T389. The downregulation of this S6K-activating phosphorylation upon mTOR modulation was always accompanied by a comparable loss of endogenous levels of phosphorylated S6 240/244 in IMR-90 cells (Fig. 2b). Furthermore, overexpression of ectopic S6K1 caused an induction of endogenous phosphorylation of S6 at 240/244 (Fig. 2c). In summary, these data confirm that the phosphorylation of S6 S240/244 is under the control of the described TSC2/mTOR/S6K cascade (Fig. 1) in the here used primary non-transformed, non-immortalized human diploid IMR-90 fibroblasts. These findings allowed the usage of these primary cells for the here described biochemical studies with the aim to avoid any pathophysiological side effects, which might originate from transformation or immortalization processes in commonly used cells, such as, e.g. HeLa.

100 nM rapamycin is known to fully block endogenous mTOR activity within several minutes (Sarbassov et al. 2006; Rosner and Hengstschläger 2008). This block of mTOR enzyme activity is represented by the accompanied disappearance of phosphorylated p70S6K1 T389, which is a major mTOR target. In the here performed experiments we used two approaches to visualize the mTOR-mediated p70S6K1 regulation upon rapamycin. First, using the antibody specific for T389-phosphorylated p70S6K1 we detected the disappearance of so phosphorylated p70S6K1 (Fig. 3a, b). In addition, using an antibody detecting total p70S6K1 protein, we found a complete shift from the slower migrating phosphorylated forms to the faster dephosphorylated p70S6K1 form upon rapamycin treatment (Fig. 3b). Strikingly, although rapamycin triggered a complete loss of the activating p70S6K1 phosphorylation within minutes, S6 phosphorylation at 240/244 remained to be detectable even after 24 h of rapamycin treatment (Fig. 3a, b). Using specific SDS-PAGE conditions we could confirm that, although remaining S6 240/244 phosphorylation is still detectable, p70S6K1 was indeed fully dephosphorylated after 24 h rapamycin treatment: the phosphorylated β, γ and δ forms totally disappeared and only the unphosphorylated p70S6K1 α form remained to be detectable (Fig. 4; compare also Chung et al. 1992). These

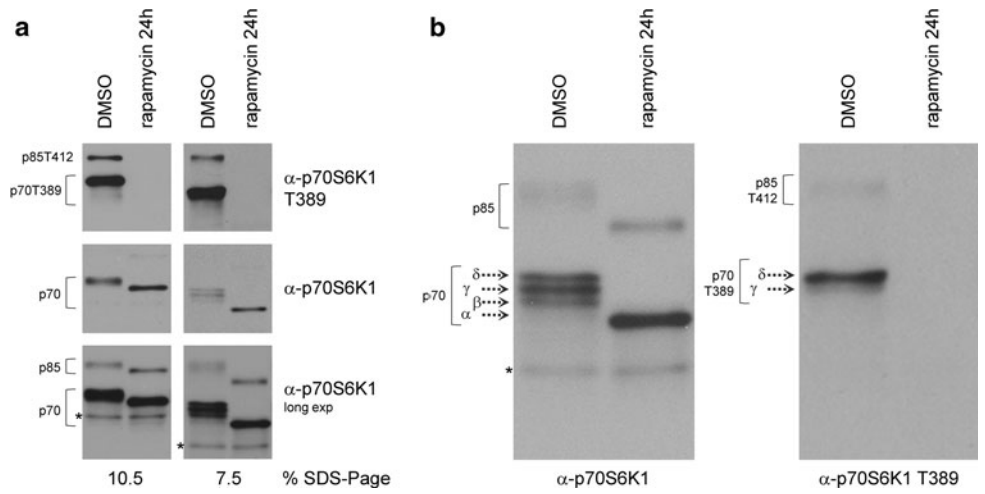

Fig. 4 Rapamycin treatment not only efficiently inhibits p70S6K1 T389 phosphorylation but also causes complete loss of phosphorylation-mediated gel shift of S6K1. **a** IMR-90 fibroblasts were treated with 100 nM rapamycin for 24 h, lysed and analysed for T389 phosphorylated p70S6K1 and total p70S6K1 via immunoblotting. To allow direct comparison of the impact of different polyacrylamide concentrations on the separation of gel shifted S6K1 forms, same lysates were resolved by SDS-PAGE using two different concentrations as indicated. *Asterisks* indicate non-specific bands. **b** For better visualization of the phosphorylation-mediated S6K1 gel shift and identification of distinct bands duplicates of lysates from experiments described in **a** were resolved by 7.5% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were cut, separately incubated with antibodies as indicated and re-merged for the chemiluminescence detection allowing direct comparison of total p70S6K1 phosphorylated at multiple sites (α, β, γ and δ forms of p70S6K1, *left*) and p70S6K1 phosphorylated at T389 (γ and δ forms of p70S6K1, *right*). The *asterisk* indicates a non-specific band

data provide first evidence for a rapamycin-resistant, mTOR- and p70S6K1-independent phosphorylation of S6 protein at 240/244.

To investigate the question whether this rapamycin-resistant S6 240/244 phosphorylation is stimulated via serum factors, we performed serum starvation and restimulation experiments with and without prior rapamycin treatment. First, in these experiments we could show that also in serum-starved (sd) cells rapamycin treatment did not cause a complete loss of S6 240/244 phosphorylation. Furthermore, comparing the serum-starved (sd) and the 0.5 h serum-restimulated time points clearly demonstrated a strong induction of S6 phosphorylation at 240/244 upon serum induction also in rapamycin-treated cells. Taken together, we found the rapamycin-resistant S6 240/244 phosphorylation to be strongly induced upon serum-mediated re-entry into the cell cycle and to be downregulated about at the time point of the G1 to S phase transition (Fig. 5).

In summary, to our knowledge for the first time, we here report the existence of a rapamycin-resistant phosphorylation of the ribosomal protein S6 at S240/244, which is induced via serum factors in a cell cycle-dependent manner. Since rapamycin blocks mTOR and the only S6 240/244-phosphorylating enzymes, the S6Ks, are strictly mTOR-regulated (Ruvinsky and Meyuhas 2006; Meyuhas 2008), our data suggest the evidence of other S6 S240/244 phosphorylating enzymes in mammalian cells.

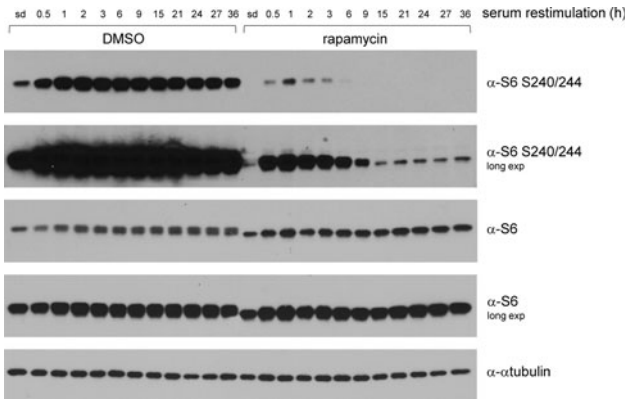

Fig. 5 Serum-mediated re-entry into the cell cycle stimulates rapamycin-resistant S6 phosphorylation at S240/244. IMR-90 fibroblasts, synchronized in G0/G1 via serum deprivation, were serum restimulated to re-enter the cell cycle in the presence or absence of rapamycin. Briefly, cells growing under full serum (10% FCS) conditions for 24–72 h were serum deprived in medium containing 0.2% serum for 48 h (sd) and serum restimulated (10% FCS) for additional 36 h. Rapamycin was added 30 min prior to restimulation at a concentration of 100 nM. Control cells were treated with an equal volume of DMSO and analysed in parallel. So treated cells were lysed and examined for the expression level of S6 S240/244, S6 and α-tubulin at indicated time points of restimulation via immunoblotting. All data presented in this figure are derived from a single (western blotting) experiment. For the proof that in this experiment rapamycin totally blocked p70S6K1 phosphorylation at all analysed time points compare other immunoblotting analyses of this experiment presented in (Rosner et al. 2009)

Finally, the finding that in addition to S6Ks, RSK phosphorylates S6 at S235/236 already raised important issues regarding the use of S6 phospho-S235/236-specific antibodies as biomarkers for activation of the mTOR pathway when staining tissue samples from tumour biopsies as has become common practice (Roux et al. 2007). Our here presented findings that there might exist an mTOR-independent rapamycin-resistant phosphorylation of S6 at 240/244 also raise concerns about the usage of S6 phospho-S240/244-specific antibodies as biomarkers for activation of the mTOR pathway in tumour development.

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