Amino Acids (2011) 40:595–600
DOI 10.1007/s00726-010-0684-2

ORIGINAL ARTICLE

Different cytoplasmic/nuclear distribution of S6 protein phosphorylated at S240/244 and S235/236

M. Rosner · C. Fuchs · H. Dolznig · M. Hengstschläger

Received: 22 June 2010 / Accepted: 29 June 2010 / Published online: 13 July 2010

Abstract Higher eukaryotic ribosome biogenesis takes place in the nucleolus and requires the import of ribosomal proteins from the cytoplasm. The ribosomal protein S6 is essential for the formation of ribosome subunits, and in mice S6 heterozygosity triggers embryonal lethality. Downstream of the mTOR (mammalian target of rapamycin) and MAPK (mitogen-activated protein kinase) signalling pathways S6 protein is phosphorylated at clustered residues S235/236 and S240/244 upon numerous physiological and pathological stimuli. Here, we show that S240/244-phosphorylated S6 is predominantly nuclear but also detectable in the cytoplasm, whereas S235/236-phosphorylated S6 is almost exclusively localized to the nucleus of primary human cells and virtually undetectable in the cytoplasm. However, in transformed cells the latter can also be detected in the cytoplasm. Experiments with the mTOR inhibitor rapamycin revealed that neither blocking the phosphorylation of S6 at S235/236 and S240/244 nor arresting the cell cycle affects the cytoplasmic/nuclear localization of S6 protein. Our findings provide new insights into the regulation of S6 phosphorylation and S6 protein localization in mammalian cells.

Keywords S6 · Posphorylation · Localization · mTOR · S6Kinase

M. Rosner · C. Fuchs · H. Dolznig · M. Hengstschläger (✉)
Institute of Medical Genetics, Medical University of Vienna, Währinger Strasse 10, 1090 Vienna, Austria
e-mail: markus.hengstschlaeger@meduniwien.ac.at

Introduction

Upon nuclear import of ribosomal proteins from the cytoplasm, ribosome biogenesis takes place in the nucleolus. Of all ribosomal proteins, it is ribosomal protein S6 (S6) that has attracted much attention. It is known to be essential for the formation of the subunits of higher eukaryotic ribosomes, it is inducibly phosphorylated and S6 heterozygosity has been demonstrated to lead to early embryonal lethality in mice (Ruvinsky and Meyuhas 2006; Meyuhas 2008). Upon translation S6 translocates to the nucleus via NLS (nuclear localization signal)-dependent mechanisms, where within the nucleoli it associates with rRNA and other proteins into premature 40S ribosomal subunits. Finally, small S6-containing ribosomal subunits are exported to the cytoplasm where they fuse to form mature ribosomes, either free floating in the cytosol or bound to the membranes of the endoplasmatic reticulum (Krüger et al. 2007; Meyuhas 2008; Fig. 1a).

In response to various growth-related stimuli S6 is dualy phosphorylated at clustered residues S235/236 and S240/244 involving the mTOR and the MAPK signalling pathways, both implicated in proliferation control, cell differentiation and cancer cell metabolism. Within the mTOR pathway PI3K (phosphatidylinositol-3-kinase) regulates the activity of the oncogenic kinase Akt (also known as protein kinase B) via PDK1 (phosphoinositide-dependent kinase-1). AKT-mediated phosphorylation of the tuberous sclerosis tumor suppressor protein tuberin (encoded by TSC2) causes its functional inactivation and leads to enhanced mTOR activity. Well known substrates of mTOR are the S6Ks (ribosomal S6 kinases), which phosphorylate 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

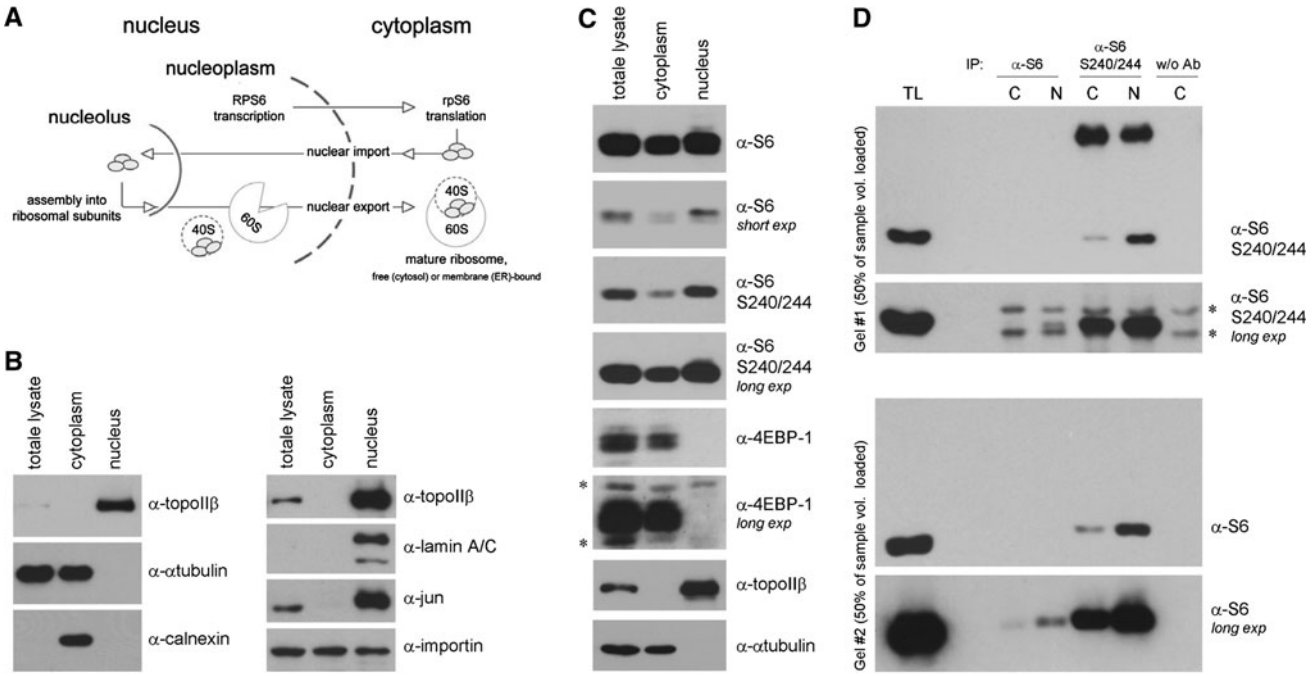

Fig. 1 S240/244 phosphorylated ribosomal protein S6 predominantly localizes to the nucleus of logarithmically growing primary IMR-90 fibroblasts. **a** Current model of the cytoplasmic/nuclear distribution of ribosomal protein S6: Upon transcription in the nucleus and translation in the cytoplasm ribosomal protein S6 translocates to the nucleus via NLS-dependent mechanisms where it accumulates in the nucleoli and associates with rRNA and other proteins to assemble into premature 40S ribosomal subunits. Following maturation in the nucleus S6-containing small ribosomal subunits are exported to the cytoplasm where they fuse to large 60S ribosomal subunits to form mature ribosomes, either free floating in the cytosol or bound to the membranes of the endoplasmatic reticulum (ER). **b** Logarithmically growing IMR-90 fibroblasts were lysed to yield total cellular protein or separated into cytoplasmic and nuclear fractions. So obtained lysates were immunoblotted with indicated antibodies to prove purity of fractions. Absence of cytoplasmic contamination of cell nuclei was proven by analysing αtubulin (cytosol) and calnexin (ER, *left panel*) and integrity of prepared cell nuclei was demonstrated by the absence of nuclear leakage into the cytoplasmic fraction by co-analysing topoII*β*, lamin A/C and jun. Additionally, the expression level of the shuttling protein importin was examined (*right panel*). **c** Lysates were prepared as described earlier and analysed for expression levels of total S6, S6 phosphorylated at S240/244, 4EBP-1, topoII*β* and αtubulin via immunoblotting. The asterisks indicate non-specific bands. **d** Equal amounts of cytoplasmic (C) and nuclear (N) fractions of IMR-90 fibroblasts were subjected to immunoprecipitation with antibodies against total S6 or S240/244 phosphorylated S6 and analysed via immunoblotting. To avoid any interference in the sequential detection of S6 and phosphorylated S6, denatured precipitates were divided into equal amounts (50:50 ratio), loaded onto separate gels and detected with antibodies as indicated. The *asterisks* indicate non-specific bands

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 (Yang and Guan 2007; Dann et al. 2007; Rosner et al. 2004; Laplante and Sabatini 2009).

On the other hand, the serine/threonine kinase Raf links Ras to the MAPK pathway. Within the MAPK cascade phosphorylation of ERK triggers activation of the kinase RSK, which is known to directly phosphorylate S6 at S235/236 via an mTOR-independent mechanism (Shaw and Cantley 2006; Ruvinsky and Meyuhas 2006; Roux et al. 2007; Meyuhas 2008; Laplante and Sabatini 2009).

Accordingly, the mTOR-regulated S6Kinases, S6K1 and S6K2, have been the only proven S6 S240/244 phosphorylating enzymes in mammalian cells. In time course experiments with the mTOR inhibitor rapamycin we recently observed rapamycin-resistant phosphorylation of the ribosomal protein S6 at S240/244. Serum-restimulation experiments further demonstrated that this rapamycin-resistant S6 S240/244 phosphorylation is induced via serum factors in a cell cycle-dependent manner. These findings provided evidence for an additional S6-phosphorylating kinase not identified so far (Rosner and Hengstschläger 2010).

In the study presented here, we have analysed the cytoplasmic/nuclear localization of S6 protein phosphorylated at S235/236 or at S240/244 in primary non-transformed non-immortalized human fibroblasts. Whereas S235/236-phosphorylated S6 is more or less exclusively localized to the nucleus, S240/244-phosphorylated S6 is predominantly nuclear but also detectable in the cytoplasm. This distribution appears to be regulated in a cell type specific manner. Rapamycin experiments lead to the conclusion that neither blocking the phosphorylation of S6 at S235/236 and at S240/244 nor arresting the cell cycle affects the cytoplasmic/nuclear localization of S6 protein. These new findings

are of relevance for a better understanding of the regulation of S6 phosphorylation and S6 protein localization in mammalian cells.

Materials and methods

Cell culture

IMR-90 primary fibroblasts, HEK293 adenovirus-transformed human embryonic kidney cells and NIH3T3 immortalized mouse fibroblasts were obtained from the American Type Culture Collection (ATCC) and 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. IMR-90 cells (ATCC #CCL-186) are fetal lung-derived, non-transformed, non-immortalized human diploid fibroblasts with finite lifetime and were obtained at passage number 10 (population doubling 25). To avoid potential effects due to cellular senescence, IMR-90 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.

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.

For cytofluorometric DNA analyses cells were fixed by rapid submersion in ice-cold 85% ethanol. After overnight fixation at −20°C, DNA was stained with 0.25 mg/ml propidium iodide, 0.05 mg/ml RNase, 0.1% Triton X-100 in citrate buffer, pH 7.8. DNA distribution of a total of 2×10^4 to 4×10^4 cells per sample was analysed on a Beckton Dickinson FACScan (Rosner and Hengstschläger 2008; Rosner et al. 2010).

Total protein extraction and cytoplasmic/nuclear fractionation

Extracts of cellular total protein containing both, cytoplasmic and nuclear proteins, were prepared by physical disruption of cell membranes by repeated freeze&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.

For cytoplasmic and nuclear fractionation cell pellets were lysed in five packed cell volume buffer F1 containing 20 mM Tris, pH 7.6, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 2 mM $MgCl_2$ and 1 mM PMSF supplemented with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.3 μg/ml benzamidinchlorid, 10 μg/ml trypsininhibitor) for 2 min at room temperature and subsequent incubation on ice for 10 min. Thereafter, NP-40 was added at a final concentration of 1% (v/v), and lysates were homogenized by passing through a 20-gauge needle for three times. Nuclei were pelleted by centrifugation at 600 g for 5 min at 4°C and supernatant containing cytoplasmic proteins was collected and stored at −80°C. Remaining nuclei were washed three times in buffer F1 containing 1% NP-40. During the last wash nuclei were stained with trypan blue and microscopically examined for number, purity and integrity. The nucleic pellets were lysed in buffer containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4, and 0.5 mM DTT, supplemented with protease inhibitors by repeated freezing and thawing. Supernatants containing soluble nucleic proteins were collected by centrifugation at 15,000 rpm for 20 min and stored at −80°C (Rosner and Hengstschläger 2007; Rosner and Hengstschläger 2008).

Immunoprecipitation and immunoblotting

For immunoprecipitation, crude cell extracts (350 μg of cytoplasmic or nuclear fractions) were diluted in NP-40-containing total cell lysis buffer and precleared with 30 μl protein A/G-Sepharose beads (Pierce) for 30–60 min at 4°C. Indicated primary antibodies were added to the cleared lysates and incubated with constant rotation for 12–16 h (overnight) at 4°C. 30 μl of a 50% slurry of protein A/G Sepharose was then added and the incubation continued for another 90 min at 4°C. So captured immunoprecipitations were washed four times with lysis buffer containing 50 mM Tris·Cl, pH 8.0, 1% NP-40, 150 mM NaCl, 10 mM *b*-glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4, and 0.2 mM PMSF supplemented with protease inhibitors aprotinin, leupeptin, benzamidinchlorid and trypsin inhibitor as described earlier. The final washing step was carried out in wash buffer containing 10 mM Tris pH 7.5 and 0.1% NP-40. Immunoprecipitated proteins were then denatured and separated from the Sepharose beads by adding SDS-sample buffer and boiling for 5 min (Rosner et al. 2007). For immunoprecipitation, antibodies specific for the following proteins were used: mouse anti-S6 (Cell Signaling, #2317) and rabbit anti-S6 S240/244 (Cell Signaling, #2215).

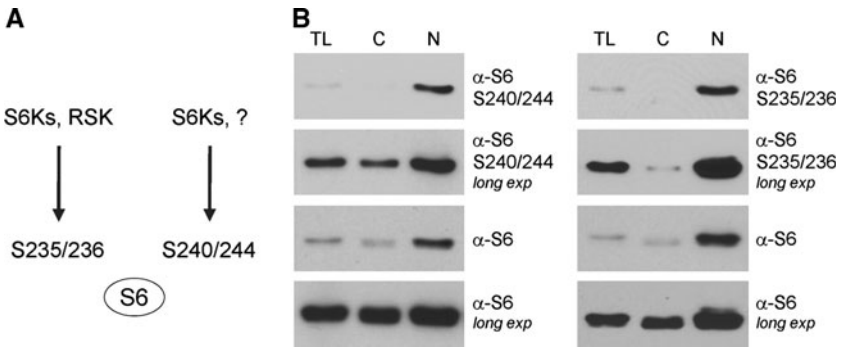

Fig. 2 S6 phosphorylated at S240/244 and S235/236 is differently distributed between the cytoplasm and the nucleus. **a** Schematic presentation of the S6 phosphorylating kinases identified so far. S6 is dually phosphorylated at clustered residues S235/236 and S240/244 by S6Ks and RSK involving both, mTOR-dependent and -independent mechanisms. Recent findings demonstrate a cell cycle-dependent but rapamycin-resistant S6 phosphorylation at S240/244 providing evidence for the existence of an additional S6-phosphorylating kinase not identified so far (Rosner and Hengstschläger 2010). **b** Total lysates (TL) and equal amounts of cytoplasmic (C) and nuclear (N) fractions derived from a single IMR-90 fractionation experiment were separately analysed for the expression levels of either S6 phosphorylated at S240/244 and total S6 (*left panel*) or S6 phosphorylated at S235/236 and total S6 (*right panel*) via immunoblotting

Denatured samples prepared from total lysates or cytoplasmic/nuclear fractions and immunoprecipitated proteins were resolved by 10 or 12.5% 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: topoisomerase IIβ (40, Transduction Laboratories, #611492), α-tubulin (DM1A, Calbiochem, #CP06), calnexin (37, Transduction Laboratories, #610524), lamin A/C (14, Transduction Laboratories, #612162), jun (3, Transduction Laboratories, #610326), importin (or karyopherin α, 2, Transduction Laboratories, #610486), S6 (54D2, Cell Signaling, #2317), S6 S240/244 (Cell Signaling, #2215), S6 S235/236 (2F9, Cell Signaling, #4856) and 4EBP-1 (Cell Signaling, #9452). Rabbit monoclonal and polyclonal antibodies were detected using anti-rabbit IgG, a HRP-linked heavy and light chain antibody from goat (A120-101P, Bethyl Laboratories); mouse monoclonal antibodies were detected using anti-mouse IgG, a HRP-linked heavy and light chain antibody from goat (A90-116P, Bethyl Laboratories); Signals were detected with the enhanced chemiluminescence method (Pierce).

Results and discussion

Logarithmically growing non-transformed non-immortalized IMR-90 human fibroblasts were separated into cytoplasmic and nuclear fractions as described previously (Rosner and Hengstschläger 2008). Purity of so obtained fractions was proven by analysing the protein levels of topoIIβ (topoisomerase IIβ), lamin A/C and c-jun (nuclear proteins), αtubulin (cytoplasmic protein), calnexin (protein specifically localized to the endoplasmatic reticulum) and importin (cytoplasmic/nuclear transport protein). Total

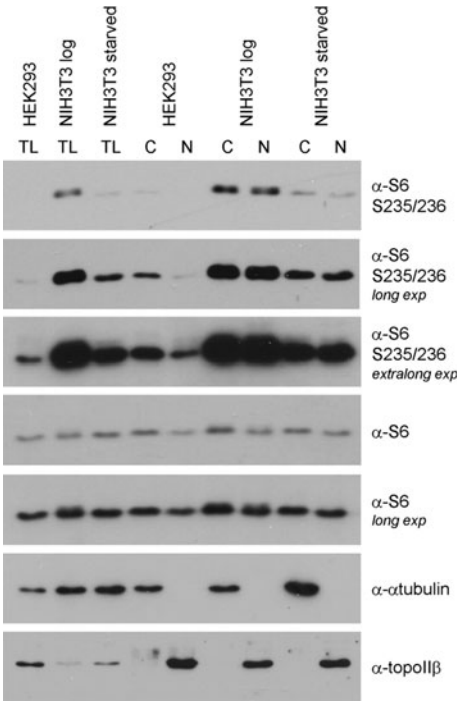

Fig. 3 Cytoplasmic/nuclear distribution of S6 phosphorylated at S235/236 is cell type-dependent. Total lysates (TL) and subcellular (C, N) fractions of logarithmically growing HEK293 and NIH3T3 cells and of G0/G1-synchronized NIH3T3 cells deprived of serum (0.5%) for 48 h were analysed for the expression levels of total S6 and S6 phosphorylated at S235/236. Purity of fractions was proven by co-analysing αtubulin and topoIIβ

lysates from the same pool of cells were analysed in parallel. In terms of purity, the fractionation experiments presented in Fig. 1b are representative for all the experiments performed in this study. These findings allowed the usage of these primary cells for the localization studies described here with the aim to avoid any pathophysiological side

effects, which might originate from transformation or immortalization processes.

Using this fractionation approach both, Western blot analyses of endogenous protein levels and immunoprecipitations, demonstrated that S6 total protein and S240/244-phosphorylated S6 are predominantly localized to the nucleus. However, significant levels of both can also be detected in the cytoplasm (Fig. 1c, d). Interestingly, 4EBP1, another well-characterized mTOR target, which is assumed to act as a translational repressor by binding and inhibiting the eukaryotic translation initiation factor (eIF4E) (Yang and Guan 2007; Laplante and Sabatini 2009), was found to be localized exclusively to the cytoplasm under the same experimental conditions (Fig. 1c).

The S6Ks phosphorylate S6 at both, S235/236 and S240/244, whereas RSK only targets S6 at S235/236. Furthermore, we recently provided evidence for an additional S6 S240/244-phosphorylating kinase not identified so far (Shaw and Cantley 2006; Ruvinsky and Meyuhas 2006; Roux et al. 2007; Meyuhas 2008; Laplante and Sabatini 2009; Rosner and Hengstschläger 2010; Fig. 2a). Accordingly, it was of interest to compare the observed cytoplasmic/nuclear localization of S240/244-phosphorylated S6 with the distribution of S235/236-phosphorylated S6 under the same experimental conditions.

From the data presented in Fig. 2b it is obvious that S240/244-phosphorylated S6 protein and S235/236-phosphorylated S6 are not equally distributed. Both phosphorylated forms were found to be predominantly localized to the nucleus. But whereas S240/244-phosphorylated S6 was also clearly detectable in the cytoplasm, S235/236-phosphorylated S6 protein was almost undetectable in the cytoplasm. To our knowledge, these data are the first to show such a different cytoplasmic/nuclear localization of the two different phosphorylated forms of S6 protein in primary human cells.

Upstream of phosphorylated S6 protein, both the mTOR and the MAPK pathway are known to be deregulated upon transformation (Shaw and Cantley 2006; Laplante and Sabatini 2009). Consequently, in the course of this study it was of interest to investigate the distribution of S235/236-phosphorylated S6 protein also in transformed cells. Whereas in non-transformed non-immortalized primary human IMR90 fibroblasts this phosphorylated form of S6

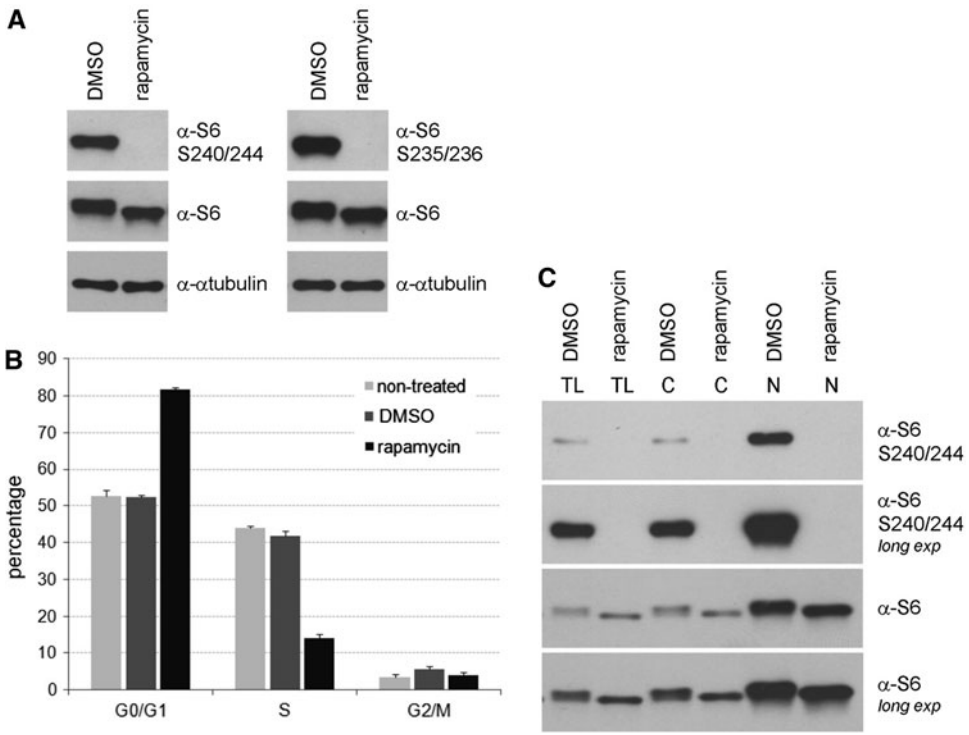

Fig. 4 Phosphorylation of ribosomal protein S6 does not alter its cytoplasmic/nuclear localization. **a** Logarithmically growing IMR-90 fibroblasts were treated with 100 nM rapamycin for 24 h, lysed and analysed for either S6 phosphorylated at S240/244 and total S6 (*left panel*) or S6 phosphorylated at S235/236 and total S6 (*right panel*). Control cells were treated with an equal volume of DMSO and analysed in parallel. **b** Cells treated as described in **a** were cytofluorometrically analysed for DNA distribution. The percentage of cells in G0/G1, S and G2/M is indicated. Cells without any prior treatment (non-treated) were co-analysed as a control. **c** Rapamycin-treated IMR-90 fibroblasts as described earlier were separated into cytoplasmic (C) and nuclear (N) fractions and examined for the expression levels of S6 phosphorylated at S240/244 and total S6 via immunoblotting

protein was nuclear (Fig. 2b), in adenovirus-transformed human HEK293 cells S235/236-phosphorylated S6 was predominantly cytoplasmic (Fig. 3). Immortalized murine NIH3T3 fibroblasts exhibited an equal distribution of S235/236-phosphorylated S6 protein between the nucleus and the cytoplasm (Fig. 3). Taken together, these findings demonstrate that the cytoplasmic/nuclear localization of S235/236-phosphorylated S6 protein varies between different cell types, what might be associated with the processes of transformation and/or immortalization. These data warrant a more detailed investigation of the cytoplasmic/nuclear localization of components of the mTOR and the MAPK pathways with the aim to clarify whether deregulated localization could be of relevance for the regulation of transformation or immortalization in mammalians.

The results obtained so far could suggest that phosphorylation of S6 might be involved in the regulation of its cytoplasmic/nuclear localization. 100 nM rapamycin is known to fully block endogenous mTOR activity within several minutes (Sarbassov et al. 2006; Rosner et al. 2009). Under the here used experimental conditions in IMR90 cells this block of mTOR enzyme activity was accompanied by a block of phosphorylation of S6 protein at both, S240/244 and S235/236 (Fig. 4a). In addition, using an antibody detecting total S6 protein, we found a complete shift from the slower migrating phosphorylated forms to the faster dephosphorylated S6 form upon rapamycin treatment (Fig. 4a). In addition, in agreement with already published data (Rosner et al. 2009) such a rapamycin treatment caused a significant cell cycle arrest of IMR90 cells represented by an increase of the amount of cells in the G0/G1 phase of the cell cycle (Fig. 4b). However, interestingly, neither the downregulation of phosphorylation of the investigated sites nor the cell cycle arrest had any effects on the cytoplasmic/nuclear localization of total S6 protein (Figs. 3, 4c).

In summary, to our knowledge for the first time, we here report that although S6 protein phosphorylation is cell-cycle regulated and is differently distributed between the cytoplasm and the nucleus, it is not involved in the regulation of the localization of total S6 protein. Our data also warrant further investigation to clarify whether the here observed cell type-specific differences of the localization of phosphorylated S6 protein might be of relevance regarding the use of phospho-specific S6 antibodies as biomarkers for activation of the mTOR or the MAPK pathway when staining tissue samples from tumor biopsies, as has become common practice (Roux et al. 2007).

Acknowledgments Research in our laboratory is supported via grants from the Herzfelder′sche Familienstiftung and from the Österreichische Nationalbank.

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