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ORIGINAL ARTICLE

p70 S6K1 nuclear localization depends on its mTOR-mediated phosphorylation at T389, but not on its kinase activity towards S6

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Abstract The protein kinase p70 S6K1 is regulated in response to cytokines, nutrients and growth factors, and plays an important role in the development of a variety of human diseases. Mammalian target of rapamycin (mTOR) is known to phosphorylate and thereby activate p70 S6K1. p70 S6K1 phosphorylates different cytoplasmic and nuclear substrates involved in the regulation of protein synthesis, cell cycle, cell growth and survival. Recently, we have shown that mTOR-mediated phosphorylation of p70 S6K1 at T389 also regulates its nucleocytoplasmic localization. Since this phosphorylation is associated with its kinase activity the question whether p70 S6K1 phosphorylation or kinase activity is essential for its proper localization remained elusive. Recently, the chemical compound PF-4708671 has been demonstrated to block p70 S6K1 kinase activity while inducing its phosphorylation at T389. This potential of PF-4708671 to separate p70 S6K1 activity from its T389 phosphorylation allowed us to demonstrate that the proper nucleocytoplasmic localization of this kinase depends on its mTOR-mediated phosphorylation but not on its kinase activity. These findings provide important insights into the regulation of p70 S6K1 and allow a more detailed understanding of subcellular enzyme localization processes.

Keywords Kinase · mTOR · p70 S6Kinase · Phosphorylation · S6

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Introduction

In mammalian cells, S6K1 is encoded by the *RPS6KB1* gene. Alternative translation generates two isoform 1 S6K1 proteins: p85 S6K1 and p70 S6K1, which is identical to p85 S6K1 but lacks its first 23 amino acids (Meyuhas and Dreazen 2010). In addition, mammalian cells express p31 S6K1 which is a second S6K1 isoform spanning 316 amino acids generated by the alternative splicing protein SF2/ASF (Karni et al. 2007; Fenton and Gout 2011).

S6K substrates include, e.g. the cytoplasmic proteins eukaryotic elongation factor 2 kinase, eukaryotic translation initiation factor 4B, BAD, insulin receptor substrate, and mTOR (which is also located in the nucleus) and the nuclear proteins cAMP-responsive element modulator τ, the 80-kDa subunit of the nuclear Cap-binding complex, and S6K1 Aly/REF-like target. An additional well-known S6K target is the ribosomal protein S6, which is dispersed throughout the cytoplasm and concentrated to the nucleoli within the nucleus, since the eukaryotic ribosomes are assembled in the nucleolus before export to the cytoplasm (Ruvinsky and Meyuhas 2006; Meyuhas and Dreazen 2010).

The best characterized upstream regulator of S6K1 is the so-called insulin signalling pathway. In this pathway, the 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 tumour suppressor protein tuberin (encoded by TSC2) causes its functional inactivation and leads to induction of the activity of mTOR, a serine/threonine kinase playing the central role within this insulin signalling cascade by regulating a wide variety of different cellular functions. In mammalian cells, two structurally and functionally distinct mTOR-containing complexes have been identified. mTORC1 is composed of

mTOR, raptor, and mLST8 and mTORC2 contains mTOR, rictor, mLST8 and sin1 (Yang and Guan 2007; Wang and Proud 2009; Sengupta et al. 2010). mTORC2 phosphorylates and activates Akt. Besides the eukaryotic initiation factor 4E-binding protein, the best characterized phosphorylation targets and downstream effectors of mTORC1 are the S6K1 proteins (Ruvinsky and Meyuhas 2006; Shaw 2008).

In a very recent study, we have demonstrated that p85 S6K1 is cytoplasmic, p70 is found in both compartments, and p31 is exclusively nuclear. We further proved that mTOR regulates the localization of p70, but not of p85 and p31. mTOR-dependent phosphorylation of p70 S6K1 at T389 is essential for its nuclear localization, which is growth factor dependent and strictly regulated during the cell cycle (Rosner and Hengstschläger 2011).

In the past the mTOR-mediated phosphorylation status of p70 S6K1 at T389 has been found to always correlate with p70 S6K1 kinase activity as evaluated by both, in vitro and in vivo experiments on the phosphorylation of ribosomal protein S6 (Holz et al. 2005; Holz and Blenis, 2005; Julien et al. 2010; Rosner and Hengstschläger, 2011). Accordingly, our data that mTOR mediated phosphorylation of p70 S6K1 at T389 is essential for its proper nucleocytoplasmic localization did not allow to separate the role of its phosphorylation and kinase activity for this regulation. Very recently, PF-4708671 a novel and highly specific inhibitor of p70 S6K1 has been identified (Pearce et al. 2010). Since S6K functions are commonly deregulated in human diseases, the development of such inhibitors not only allows more detailed investigations of these enzymes but also the effective targeting of deregulated S6K signalling in patients (Fenton and Gout 2011). Interestingly, treating cells with PF-4708671 suppresses the activity of p70 S6K1 to phosphorylate its substrates, but also induces the phosphorylation of p70 S6K1 at T389 (Pearce et al. 2010). To the best of our knowledge, this is the first description of decoupled T389 phosphorylation and p70 S6K1 kinase activity.

In this study, we made use of PF-4708671 to investigate whether p70 S6K1 phosphorylation or kinase activity is essential for its nucleocytoplasmic localization. Studying non-transformed non-immortalized primary human IMR-90 fibroblasts, we confirmed that this inhibitor not only induces p70 S6K1 phosphorylation at T389 but also decreases p70 S6K1 in vivo kinase activity towards S6. Using the mTOR inhibitor rapamycin and performing mTOR-specific siRNA knockdown experiments showed that this PF-4708671-mediated induction of p70 S6K1 phosphorylation at T389 is mTOR-dependent. Cytoplasmic and nuclear fractionation experiments demonstrated that p70 S6K1 localization is strictly regulated by its T389 phosphorylation independent of its kinase activity. The relevance of these data for a better understanding of both p70 S6K1 regulation and subcellular enzyme localization, is discussed.

Materials and methods

Cell culture and reagents

Primary 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 2 mM L-glutamine at 37°C and 5% CO_2. To avoid potential effects due to cellular senescence, cells were grown for not more than 9 additional passages ($\leq$48 total population doublings) and were regularly analysed by standard karyotyping to confirm a normal diploid karyotype (Rosner and Hengstschläger 2011). Experiments involving the S6K1 inhibitor PF-4708671 (Pfizer, Sigma) or the mTOR inhibitor rapamycin (Calbiochem) were performed in the absence (DMSO vehicle control) or presence of the drug at a final concentration of 10 μM or 100 nM, respectively, unless otherwise indicated. To study the effects of inhibitor-treatments under various growth conditions, cells were either grown in complete growth medium supplemented with 10% serum (maintenance conditions, *+serum*) or were deprived of serum in growth medium containing 0% serum for 18 h (starvation conditions, *−serum*) with or without subsequent restimulation with 100 ng/ml rhIGF-1 (Calbiochem) (*−serum/IGF-1 restimulation*) or 10% serum (*−serum/serum restimulation*) for 30 min.

RNA interference

siRNA transfection experiments were performed using Lipofectamine RNAiMAX transfection reagent (Invitrogen) following the guidelines provided by the manufacturer. Pooled siRNAs specifically targeting human raptor, rictor, mTOR or S6K1 (ON-TARGET*plus SMART* pool reagents, Dharmacon) were delivered to the cells at a final concentration of 50 nM. A pool of four non-targeting siRNAs was used as a control for non-sequence-specific effects (Rosner et al. 2010).

Preparation of whole cell lysates and cytoplasmic/nuclear fractions

Whole cell lysates containing both, cytoplasmic and nuclear proteins, were prepared by physical disruption of

the 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, 25% glycerol, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.5 mM NaF,and 0.5 mM Na_3VO_4 supplemented with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0,3 μg/ml benzamidinchlorid, 10 μg/ml trypsin inhibitor by freezing and thawing. Supernatants were collected by centrifugation at 20,000 g for 20 min at 4°C. For cytoplasmic and nuclear fractionation cell pellets were lysed in 5-packed cell volume buffer F1 containing 20 mM Tris, pH 7.6, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM PMSF supplemented with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 0,3 μg/ml benzamidinchlorid, and 10 μg/ml trypsin inhibitor) 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 or equivalent through a 200-μl tip 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. Pellets of pure nuclei were lysed in buffer containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4, and 0.5 mM DTT,and supplemented with protease inhibitors by repeated freezing and thawing. Supernatants containing soluble nucleic proteins were collected by centrifugation at 20,000 g for 20 min (Rosner and Hengstschläger 2011).

Immunoblotting

Equal amounts (5–15 μg) of denatured samples prepared from whole cell lysates or subcellular fractions (cytoplasmic/nuclear ratio of loaded protein amount = 1:1) were resolved by 11% SDS–PAGE and transferred to nitrocellulose. For immunodetection, antibodies specific for the following proteins were used: p70S6K (clone 49D7), N-term (#2708, Cell Signaling), p70S6K T389 (clone 108D2) (#9234, Cell Signaling), S6 ribosomal protein (clone 54D2) (#2317, Cell Signaling), S6 ribosomal protein

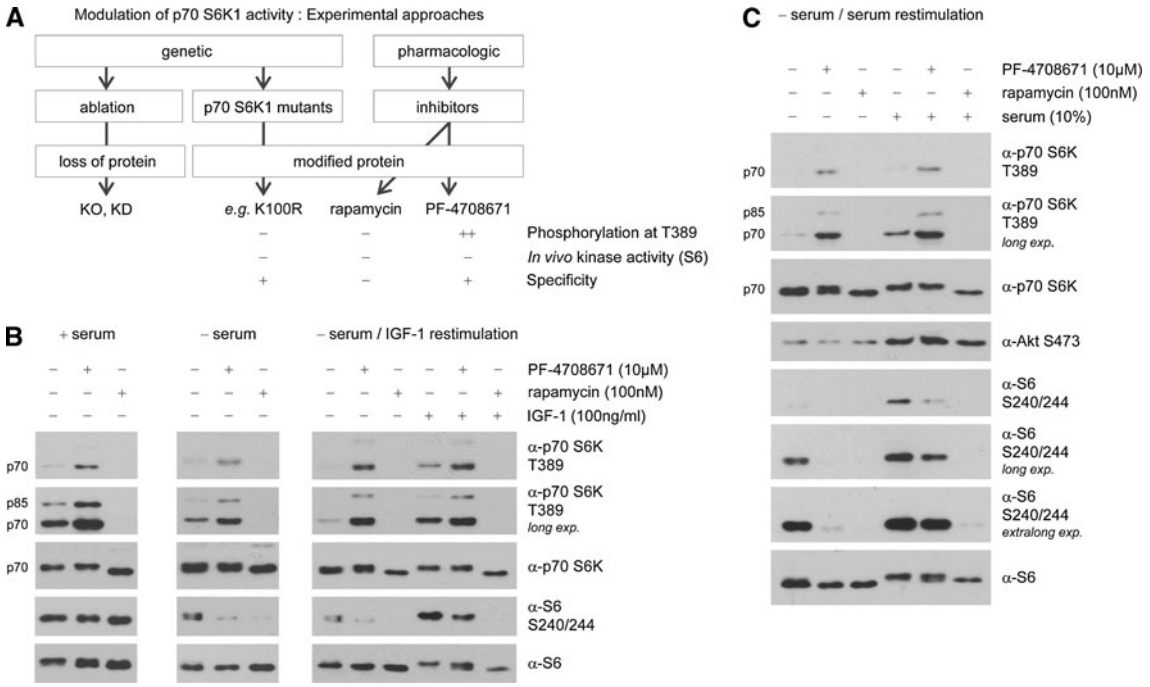

Fig. 1 PF-4708671-mediated decoupling of p70 S6K1 phosphorylation at T389 and its in vivo kinase activity towards ribosomal protein S6 under various growth conditions. **a** Comparative schematic outline of different, commonly used experimental tools to modulate p70 S6K1 activity (*KO* knockout, *KD* knockdown, *K100R* mutated form of p70S6K1 containing a Lysine^{100}→ Arginine mutation, kinase dead). **b** Primary IMR-90 fibroblasts, either grown under full serum conditions (10% serum for 16 h; *+serum, left panel*) or deprived of serum (0% serum for 18 h; *−serum, middle panel*), were treated with 10 μM PF-4708671 or 100nM rapamycin for 40 min and analysed for phosphorylated and total forms of p70 S6K1 and S6 via immunoblotting. Serum deprived cells pretreated with PF-4708671 or rapamycin for 30 min and restimulated with 100 ng/ml IGF-1 for another 30 min were analysed in the same way (*−serum/IGF-1 restimulation, right panel*). **c** Experiments were performed as described in *B, right panel* except that 10% serum was used for restimulation. Whole cell lysates of so treated cells were immunoblotted with antibodies against phosphorylated and/or total forms of p70 S6K1, Akt and ribosomal protein S6

S240/244 (#2215, Cell Signaling), Akt S473 (clone D9E) (#4060, Cell Signaling), mTOR (#2972, Cell Signaling), raptor (#A300-506A, Bethyl Laboratories), rictor (#A300-459A, Bethyl Laboratories), α-tubulin (clone DM1A) (#CP06, Calbiochem) and fibrillarin (clone C13C3) (#2639, Cell Signaling). Rabbit polyclonal and monoclonal antibodies were detected using anti-rabbit IgG, a HRP-linked heavy and light chain antibody from goat (#A120-101P, Bethyl Laboratories, Montgomery, TX, US); 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) (Rosner and Hengstschläger 2011).

Results and discussion

We have recently reported that mTOR-mediated phosphorylation of p70 S6K1 at T389 is regulating its nucleocytoplasmic localization (Rosner and Hengstschläger 2011). However, since phosphorylation and kinase activity were always found to be coupled (Holz et al. 2005; Holz and Blenis, 2005; Julien et al. 2010; Rosner and Hengstschläger, 2011) their specific individual roles for p70 S6K1 localization remained elusive. In the past, different approaches, including the generation of knock-out mice, siRNA experiments, the ectopic expression of loss of function mutants (e.g. kinase dead or TOS motif mutated forms of p70 S6K1) or treatment with the mTOR inhibitor rapamycin, were used to modulate the activity of p70 S6K1. However, none of these approaches allows the separate investigation of T389 phosphorylation and p70 S6K1 kinase activity for specific p70 S6K1 regulations (Fig. 1a). Very recently, the kinase inhibitor PF-4708671 has been shown to induce T389 phosphorylation, but to block p70 S6K1 kinase activity (Pearce et al. 2010). To the best of our knowledge, this is the first description of an experimental tool that allows to separately analyse these two events for their relevance for p70 S6K1 regulations (Fig. 1a).

For the above mentioned study on p70 S6K1 nucleocytoplasmic localization, we have used primary IMR-90 fibroblasts, because they are non-transformed, non-immortalized, cytogenetically normal and stable, and commercially available to all the colleagues in the field. These cells can be grown with reasonable proliferation rates, can be cell cycle-synchronized via serum deprivation and restimulation, and can be used to perform nucleocytoplasmic fractionations of high purity (Rosner and Hengstschläger 2010, 2011). Accordingly, as a first step we here wanted to confirm the described biochemical properties of PF-4708671 in IMR-90 cells. Indeed, we found this inhibitor to induce p70 S6K1 T389 phosphorylation in IMR-90 cells grown in 10% serum (maintenance conditions), under 0% serum starvation conditions and under 0% serum starvation conditions with subsequent short term restimulation with IGF-1 or serum (restimulation conditions) (Figs. 1b, c). It is important to note, that we never observed induction of apoptosis under the chosen experimental conditions (data not shown). In perfect agreement with the findings in HEK-293 cells reported earlier (Pearce et al. 2010), we found PF-4708671 to downregulate the p70 S6K1 activity to phosphorylate S6 at S240/244 with the most pronounced effects observed under conditions of serum deprivation. The same experimental approach, namely incubation of IMR-90 cells with PF-4708671, triggers both, an induction of p70 S6K1 T389 phosphorylation and a downregulation of its in vivo kinase activity towards S6 (Fig. 1b, c). These data confirm that this inhibitor is a powerful tool to separately investigate the role of the p70 S6K1 phosphorylation status and kinase activity in primary human cells.

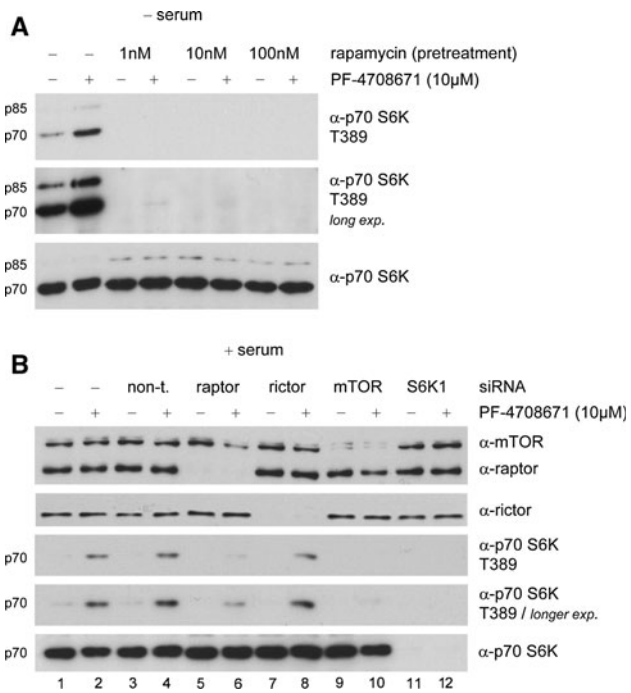

Fig. 2 PF-4708671-mediated induction of p70 S6K1 phosphorylation at T389 depends on mTOR/raptor. **a** Serum deprived IMR-90 fibroblasts were pretreated with indicated concentrations of rapamycin for 30 min and were further incubated with 10 μM PF-4708671 for 40 min. Whole cell lysates were investigated for expression levels of p70 S6K1 and p70 S6K1 phosphorylated at T389 via immunoblotting. **b** Cells transfected with siRNAs as indicated (*lanes 3–12, non*-t non-targeting control) or left untreated (*lanes 1 and 2*) were replated at 72 h after transfection and were further incubated under full serum conditions for 16 h. Thereafter, PF-4708671 was added at a concentration of 10 μM for 40 min and whole cell lysates were prepared. Immunoblot analyses of mTOR, raptor, rictor and phosphorylated and total forms of p70 S6K1 are presented

Since the T389 phosphorylation of p70 S6K1 is mediated by mTOR (Yang and Guan 2007; Wang and Proud 2009), we wanted to study whether the PF-4708671 effects on this phosphorylation are mTOR-dependent. Pretreatment of IMR-90 cells with low doses of rapamycin and subsequent incubation with PF-4708671 clearly demonstrated the PF-4708671-mediated induction of T389 phosphorylation to be mTOR dependent (Fig. 2a). This relevance of mTOR for the PF-4708671-mediated induction of p70 S6K1 phosphorylation was additionally confirmed in experiments with siRNA-mediated knockdown of endogenous mTOR (Fig. 2b).

Since in mammalian cells mTOR exists in two distinct protein complexes [with raptor and mLST8 in mTORC1 and with rictor, mLST8 and sin1 in mTORC2; (Wang and Proud 2009; Sengupta et al. 2010)], we next wanted to clarify the relevance of these complexes for PF-4708671-mediated induction of p70 S6K1 phosphorylation. Therefore, we specifically modulated mTORC1 and mTORC2 via raptor-and rictor-specific siRNAs, respectively. The separate knockdown of these two endogenous mTOR protein complex components revealed mTOR/raptor (mTORC1), but not mTOR/rictor (mTORC2), to be involved in this regulation (Fig. 2b). In agreement with data reported by Pearce and colleagues, our experiments show that PF-4708671 induces p70 S6K1 phosphorylation at T389 in an mTORC1-dependent manner (Pearce et al. 2010).

To investigate the effects of PF-4708671 on p70 S6K1 nucleocytoplasmic localization, we next performed nuclear and cytoplasmic fractionation of IMR-90 cells grown under serum starvation conditions. Purity of the so-obtained fractions was confirmed by Western blot analyses of α-tubulin (cytoplasmic) and fibrillarin (nuclear) (Fig. 3).

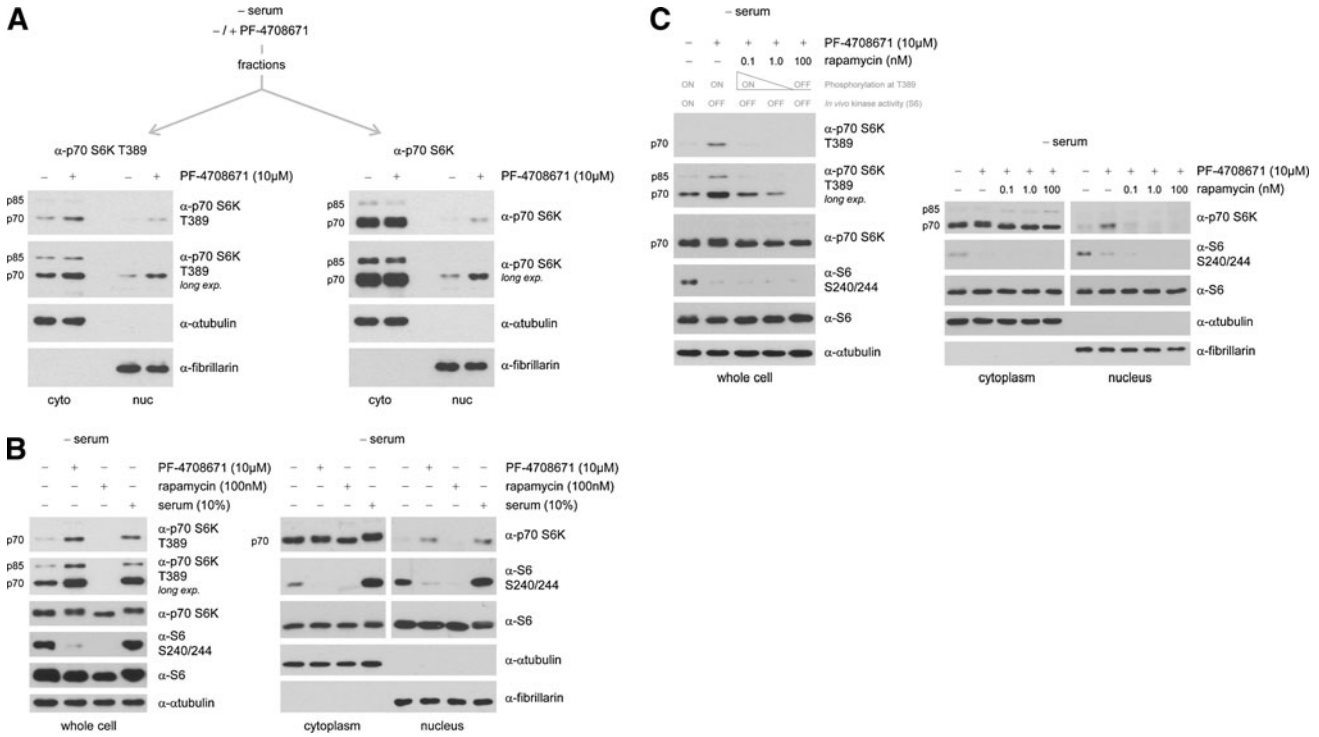

Fig. 3 p70 S6K1 nuclear localization depends on its mTOR-mediated phosphorylation at T389 but not on its kinase activity towards ribosomal protein S6. **a** Serum deprived IMR-90 fibroblasts were treated with or without 10 μM PF-4708671 for 40 min and separated into cytoplasmic and nuclear fractions. To avoid any antibody interference in the sequential immunodetection of p70 S6K1 and its T389 phosphorylated form (both detected by rabbit antibodies) same lysates were separately analysed using either an α-p70 S6K1 T389 antibody (*left panel*) or an α-p70 S6K1 antibody (*right panel*). α-tubulin and fibrillarin were co-analysed and serve as fractionation and loading controls. **b** Whole cell lysates (*left panel*) and cytoplasmic and nuclear fractions (*right panel*) of serum deprived IMR-90 fibroblasts treated with indicated concentrations of PF-4708671, rapamycin or serum for 40 min were analysed for total and phosphorylated forms of p70 S6K1 and S6 via immunoblotting. α-tubulin and fibrillarin were co-analysed and serve as fractionation and loading controls. **c** Serum deprived IMR-90 fibroblasts were either incubated with 10 μM PF-4708671 alone or were simultaneously incubated with 10 μM PF-4708671 and increasing amounts of rapamycin as indicated for 40 min. Whole cell lysates (*left panel*) and subcellular fractions (*right panel*) were prepared and investigated for expression levels of phosphorylated and total forms of p70 S6K1 and S6 via immunoblotting. α-tubulin and fibrillarin were co-analysed. For immunoblot analyses presented in (**b** and **c**) it is important to note that whole cell lysate and corresponding subcellular fractions were always prepared from the same pool of cells and therefore represent a single experiment under exactly the same conditions

Serum treatment, accompanied by an induction of both, T389 phosphorylation and p70 S6K1 kinase activity towards S6 [additionally analysed by the earlier described nuclear phosphorylation status of S6 240/244; (Rosner et al. 2011)], causes a prominent upregulation of nuclear p70 S6K1 protein levels (Fig. 3a, b). Although PF-4708671 triggers downregulation of p70 S6K1 activity towards S6, it still induces the nuclear localization of this kinase. The latter was accompanied by a strong induction of p70 S6K1 phosphorylation at T389 (Fig. 3a, b). These data demonstrate for the first time that the phosphorylation at T389 but not the kinase activity of p70 S6K1 is regulatively involved in the nucleocytoplasmic localization of p70 S6K1. This notion was further confirmed by our finding that blocking PF-4708671-mediated induction of T389 phosphorylation via simultaneous incubation with rapamycin also inhibited the induction of nuclear p70 S6K1. These data demonstrate that the regulation of the S6K1 localization via PF-4708671 is not a bystander effect, but rather a direct consequence of the induction of the T389 phosphorylation via this inhibitor. Cotreatment with PF-4708671 and low doses of rapamycin allowed to hold the mTOR-mediated T389 phosphorylation status at normal endogenous levels even under experimental conditions, under which this inhibitor blocks S6. These experiments clearly demonstrated that, when PF-4708671 does not affect T389 S6K1 phosphorylation, it has no effects on the nucleocytoplasmic localization of S6K1 even when the S6K1 activity against S6 is blocked (Fig. 3c).

Taken together, we identified phosphorylation of p70 S6K1 at T389 to regulate p70 S6K1 localization independently of its kinase activity. On the one hand, these data provide new insights into the regulation of the nucleocytoplasmic regulation of p70 S6K1. On the other hand our findings prove PF-4708671 to be a very useful tool for basic research. It is a specific inhibitor of p70 S6K1 (Pearce et al. 2010), which also allows to separately investigate the relevance of T389 phosphorylation and kinase activity for p70 S6K1 regulations. Indirect targeting of S6K and other mTORC1 targets via rapamycin analogues is clinically used in cancer treatment (Yuan et al. 2009; Fenton and Gout 2011). It has recently been argued that in cancers, where it has been shown that direct deregulation of S6K1 itself is involved, the use of specific S6K1 inhibitors might be the better option for avoiding the side effects due to the broader effects of mTORC1 inhibition (Fenton and Gout, 2011). Accordingly, it is of importance to obtain a more detailed understanding of the cellular consequences mediated by these inhibitors. In the presented study we report a more detailed investigation of the intracellular consequences of a recently characterized S6K1 inhibitor.

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