Activation of S6K1 (p70 ribosomal protein S6 kinase 1) requires an initial calcium-dependent priming event involving formation of a high-molecular-mass signalling complex

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The mitogen-stimulated protein kinase p70 ribosomal protein S6 kinase 1 (S6K1) is a key enzyme in the regulation of cell growth and proliferation. Activation of S6K1 requires a complex, ordered series of conformational changes and phosphorylation reactions. While the role of sequential, multi-site phosphorylation has been extensively detailed, characterization of the priming step required to initiate this cascade has remained elusive. In the present study we show for the first time that this priming process is dependent on calcium. Calcium-dependent regulation of S6K1 did not specifically target Thr-229 and Thr-389, the key regulatory phosphorylation sites; rather, calcium chelation resulted in a global inhibition of S6K1 phosphorylation. Mutation of individual phosphorylation sites in the auto-inhibitory and hydrophobic domains to acidic residues (to mimic phosphorylation) yields a kinase that remains sensitive to calcium chelation, while the combined mutations alleviate the requirement for calcium. Furthermore, deletion of the C-terminal residues (398–502) also renders the kinase insensitive to calcium. We hypothe-

size that the initial calcium-dependent process is required to release an inhibitory interaction between the C- and N-termini of S6K1, thus allowing phosphorylation of these key domains. The requirement for this priming step can only be overcome by mutations mimicking the phosphorylation of both the auto-inhibitory and hydrophobic domains. We further propose that the priming event involves formation of a calcium-dependent protein complex that releases the interaction between the N- and C-termini. S6K1 is then accessible for activation by the kinases that target the known regulatory phosphorylation sites. Consistent with this hypothesis, serum stimulation of S6K1 activity is associated with its incorporation into a calcium-dependent high-molecular-mass complex.

Key words: calcium, phosphoinositide-dependent kinase 1, phosphorylation, protein interactions, signal transduction, S6 kinase 1.

INTRODUCTION

Studies using molecular approaches and knockout technology have identified p70 ribosomal protein S6 kinase 1 (S6K1) as an important factor linking growth/proliferation signals to the regulation of protein synthesis and cell size [1–8]. Multiple phosphorylation of the 40 S ribosomal protein S6 by S6K1 results in a specific increase in translational efficiency for the family of essential mRNAs containing an oligopyrimidine tract at their transcriptional start site (5' TOP mRNAs). This class of mRNAs encodes many components of the protein synthetic apparatus and can represent up to 20 % of the total mRNA in the cell [9,10]. Understanding the regulation of S6K1 will have major implications not only for the control of normal cellular growth, but also for cases of abnormal growth such as cancer. In fact, a specific role for S6K1 in breast cancer has been inferred from the observation that the S6K1 gene is amplified and the protein is overexpressed in 8.8 % and 15.6 % of primary tumours respectively [11]. Recently, analogues of the S6K1 signalling pathway antagonist rapamycin have been tested in pre-clinical and phase I studies, demonstrating preliminary evidence of antitumour effects in renal cell carcinoma and non-small-cell lung cancer [12].

Extensive studies using *in vivo* ³²P labelling, phospho-specific antibodies and structure/function mutants have revealed that

S6K1 is regulated in a complex manner, involving sequential phosphorylation at multiple sites [13–16]. While the key regulatory phosphorylation sites have been identified, a number of details of the process remain to be elucidated. The current model for activation of S6K1 is illustrated in Figure 1 [17,18]. Step 1, the initial or priming step for activation, requires the release of the interaction between the S6K1 auto-inhibitory (C-terminal) and N-terminal domains. Unlike the other steps in this model, little is known about the mechanism by which this release occurs. Once achieved, four key C-terminal sites (Ser-411, Ser-418, Ser-424 and Thr-421) are phosphorylated by a yet to be identified proline-directed kinase(s) (step 2). This C-terminal sequence has been proposed to act as an auto-inhibitory domain, based on its similarity to the phosphorylation site sequence in ribosomal protein S6. Peptide analogues of the sequence inhibit S6K1 activity [19], and mutation of these sites to acidic residues, to mimic phosphorylation, results in increased basal activity [13]. Phosphorylation of these sites allows the sequential phosphorylation of Thr-389 in a hydrophobic motif that is conserved in other AGC kinases (step 3), and finally (step 4) the activating phosphorylation of Thr-229 in the activation loop. Thr-229 has been shown to be phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) *in vivo* [20]. The kinase phosphorylating Thr-389 *in vivo* remains to be definitively identified, although the mammalian target of rapamycin (mTOR) and,

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; mTOR, mammalian target of rapamycin; NP40, Nonidet P40; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; SGK, serum- and glucocorticoid-inducible kinase; S6K1, p70 ribosomal protein S6 kinase 1.

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Figure 1 Current model for S6K1 activation

Step 1: release of the interaction between the auto-inhibitory (C-terminal) and N-terminal domains of S6K1. Step 2: phosphorylation of four key C-terminal sites (Ser-411, Ser-418, Ser-424 and Thr-421) stabilizes the kinase. Step 3: phosphorylation of Thr-389 by mTOR, NEK6 (NimA-related kinase) or an as yet undefined kinase. Phosphorylation of this site is inhibited by rapamycin and Ro 31-6045 (RoV). Step 4: phosphorylation of Thr-229 by PDK1 results in full activation of S6K1.

recently, the NimA-related kinase NEK6/7 [21] have been proposed as candidate kinases [18]. Thr-389 is the major phosphorylation site to be inhibited following rapamycin treatment of cells, and its substitution with a glutamic acid residue confers rapamycin resistance on the kinase [14]. Despite intensive efforts, the signalling mechanisms responsible for the initial priming event (step 1) and the subsequent phosphorylation of the auto-inhibitory domain of S6K1 (step 2) remain to be defined.

S6K1 is a member of the AGC kinase family, which includes protein kinase C (PKC), protein kinase A (PKA), Akt, serum- and glucocorticoid-inducible kinase (SGK) and PDK1. All of the AGC family members contain regulatory phosphorylation sites analogous to Thr-389 and Thr-229 of S6K1, and hence are presumed to have related mechanisms of activation [20,22–26]. However, there are also key distinctions to allow for differential signalling. This was exemplified by our previous observation that calcium was absolutely required for activation of S6K1 by growth factors, but superfluous for the closely related kinase Akt [27]. This calcium dependence is consistent with the calcium requirement observed for cell growth and proliferation [28,29], and we hypothesized that it may provide a clue to elucidating the unique aspects of S6K1 signalling.

In order to clarify the mechanism by which calcium regulates S6K1, we have used acidic structure/function mutants of the phosphorylation sites required for steps 2 and 3 of the activation sequence. The serum-stimulated activities of wild-type S6K1 (2B4), an auto-inhibitory site acidic mutant (D3E; step 2) and a Thr-389 acidic mutant (dE; step 3) were all abolished by calcium chelation, indicating that phosphorylation of the step 2 and 3 sites was not the calcium-dependent process that is required for activation of the kinase. However, a C-terminal truncation mutant (Δ C) and a combined mutant (dED3E) that abolished steps 2 and 3 were both resistant to calcium regulation. Each of these mutants requires phosphorylation at Thr-229 (step 4) for activity, indicating that final activation by PDK1 is independent of calcium. Moreover, using biochemical analyses, we demonstrated that calcium chelation did not regulate any specific phosphorylation site within S6K1, but rather resulted in a global inhibition of phosphorylation. We concluded from these results that the first step in S6K1 activation, i.e. release of an N-

terminus–C-terminus interaction, is regulated by calcium, and that the requirement for step 1 can only be overcome by deletion of a section of the interaction domain or by circumventing both steps 2 and 3.

In addition, the calcium chelation-induced inhibition of S6K1 activity was correlated with the disruption of a growth factor-dependent high-molecular-mass complex containing the kinase. Addition of EGTA to serum-stimulated cells also disrupted pre-formed complexes, indicating that calcium is required for both complex formation and stability. We hypothesize from our data that a calcium-dependent binding protein interacts with S6K1 within a high-molecular-mass complex, thus releasing the interaction between the N- and C-termini, subsequently priming the kinase so that the activation process can begin.

EXPERIMENTAL

Materials

Ionomycin, wortmannin and rapamycin were purchased from Sigma, tissue culture reagents were from Gibco BRL, and general chemicals were from BDH.

Cell culture and preparation of cell extracts

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in a 5% CO₂ atmosphere. Cells were plated for 24 h and then made quiescent by culturing for 24 h in serum-free DMEM. Serum-starved cells were then treated at 37 °C with 20% (v/v) FBS or 1 μ M ionomycin for 20 or 10 min respectively. Alternatively, cells were pretreated with 10 mM EGTA, 200 nM wortmannin or 20 nM rapamycin for 20 min. Following stimulation, the cells were rinsed once with ice-cold PBS and lysed in lysis buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 1% (w/v) Nonidet P40 (NP40), 1 mM EDTA, 50 mM NaF, 40 mM β -glycerophosphate, 0.1 mM sodium vanadate, 1 mM benzamide and 0.5 mM PMSF. Cell extracts were collected with a plastic scraper, and cleared by centrifugation at 4 °C for 15 min at 12000 g. Aliquots of the supernatant were frozen in liquid

Figure 2 Activation of transfected S6K1 is calcium sensitive

HEK293 cells were transfected with wild-type S6K1 (2B4), treated and harvested as described in the Experimental section. Myc-tagged 2B4 was immunoprecipitated and S6K1 activity determined. The activities from four separate experiments are expressed as fold activation compared with serum-starved (SS) cells. Values are means $\pm$ S.E.M. The significance of the observed differences in enzyme activity was determined using a paired Student's *t* test: **P* < 0.05; ***P* < 0.005. SS, serum starved; S, 20% (v/v) serum; ES, pretreated with 10 mM EGTA then 20% serum; RS, pretreated with 20 nM rapamycin then 20% serum; I, pretreated with 1 μ M ionomycin.

nitrogen and stored at -70°C . The protein concentration of cell extracts was determined using the Bio-Rad DC Protein Assay with BSA as a protein standard.

Transfections and plasmids

HEK293 cells were plated at 0.75×10^6 cells per well in a six-well plate, and 24 h after plating were transfected using 3 μ l of Eugene per 2 μ g of DNA according to the manufacturer's protocol (Boehringer Mannheim) for 48 h, after which the cells were serum-starved for 24 h. Following serum starvation, cells were stimulated and harvested as described above.

The PDK1 expression construct pCMV5-PDK1 was a gift from Dr B. A. Hemmings (Friedrich Miescher Institute). The S6K1 expression constructs PRK5 GST-S6K1, PRK5 Myc-tagged 2B4 (wild type), dE, D3E, dED3E and Δ C have been described previously [14,30].

Immunoprecipitation activity assay for S6K1

Endogenous S6K1 or Myc-tagged S6K1 were immunoprecipitated by incubating 250 μ g or 80 μ g of cell extract with an antibody directed to the C-terminal region of S6K1 (anti-S6K1) [1] or to the Myc tag (9E10) [14] respectively. The sample and antibody were tumbled for 2 h at 4°C , then 30 μ l of washed Protein A-Sepharose beads (50% slurry) was added and the samples were tumbled for a further 30 min. The beads were washed at 4°C once with lysis buffer and then once with a kinase dilution buffer (50 mM Mops, pH 7.2, 10 mM *p*-nitrophenyl phosphate, 10 mM MgCl_2 and 0.1% Triton X-100). S6K1 was assayed using glutathione-tagged S6 as a substrate [31]. The significance of observed differences in enzyme activity was determined using a paired Student's *t* test.

Metabolic labelling

HEK293 cells were plated at 2×10^6 cells per 100 mm plate for 24 h, then transfected as described above. After 48 h the cells were serum starved in phosphate-free DMEM containing [^{32}P]P_i (1 mCi/5 ml) as described previously [14]. The cells were then treated and harvested, and ^{32}P -labelled S6K1 was immunoprecipitated as described above.

Tryptic phosphopeptide mapping

^{32}P -labelled S6K1 was separated on SDS/PAGE [15% (w/v) acrylamide], exposed to autoradiography, excised from the gel, and subjected to in-gel tryptic digestion [32,33]. Tryptic peptides were extracted sequentially with water and 80% (v/v) acetonitrile, and the combined extracted peptides were lyophilized. Phosphopeptides were separated by either two-dimensional mapping or HPLC followed by ascending TLC, as described previously [14]. For the two-dimensional mapping, a minimum of 500 c.p.m. of each sample was spotted on to the cellulose plate; alternatively, 50000 c.p.m. per sample was loaded for HPLC on a glass-lined C18 column (2 mm $\times$ 100 mm; SGE). Fractions of 200 μ l were collected, vacuum dried, resuspended in 5 μ l of pH 1.9 electrophoresis buffer and spotted on to a cellulose TLC plate. The plates were subjected to ascending TLC as described previously [14]. Radiolabelled phosphopeptides were visualized after a 24 h exposure using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Size-exclusion chromatography

HEK293 cells were plated at 5×10^6 cells per 150 mm plate for 24 h, then serum starved and treated as described. Samples were harvested in lysis buffer minus EDTA but containing 0.1% (w/v) NP40, and cleared by centrifugation at 20000 *g* for 30 min. A sample containing 700 μ g of protein was chromatographed at 40 μ l/min in lysis buffer without calcium chelators or NP40 on a Superdex 200 gel filtration column (PL 3.2/30; Amersham Pharmacia Biotech AB) attached to a SMART system. Samples of 80 μ l were collected. The column was calibrated using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and albumin (67 kDa) as molecular mass standards.

Immunoprecipitation of PDK1 and Western blotting of gel filtration samples

The gel filtration fractions or endogenous PDK1 immunoprecipitated from pooled gel filtration fractions were separated by SDS/PAGE. Endogenous PDK1 immune complexes were formed by tumbling three pooled gel filtration fractions for 2 h at 4°C , the sample and 10 μ l of anti-PDK1 antibody (Upstate Biotechnology); 30 μ l of washed Protein A-Sepharose beads (50% slurry) were then added and the sample was tumbled for a further 30 min. The beads were washed twice with lysis buffer and then boiled in Laemmli sample buffer. The samples were separated on SDS/PAGE (10% or 15% gel) and transferred to PVDF membranes (Immobilon-P; Millipore). Membranes were incubated in PBS containing 0.5% Tween 20 (PBST) and 5% (w/v) dried milk (PBSM) for 1 h. After blocking, the membranes were probed with 1:2000 dilutions of anti-S6K1, anti-PDK1 (Upstate Biotechnology), anti-PKC ζ (Santa Cruz), anti-PKC α (Santa Cruz) or anti-Rac1 (Transduction Laboratories) antibodies in PBSM. The membranes were then washed with PBST and incubated for 1 h with 1:1000 dilutions of an anti-rabbit,

-sheep or -mouse secondary antibody conjugated to horseradish peroxidase. Bound antibodies were detected with the enhanced chemiluminescence system (Perkin Elmer).

RESULTS

Activation of exogenously expressed S6K1 is calcium sensitive

In order to delineate the calcium-sensitive step of S6K1 activation (see Figure 1), we first confirmed that transfected Myc-tagged S6K1, transiently expressed in HEK293 cells, behaved in a manner similar to that of endogenous S6K1. Serum stimulated transfected wild-type S6K1 (2B4) activity 1.6-fold, and this stimulation was inhibited by pretreatment with 10 mM EGTA to levels as low as those obtained in the presence of rapamycin, the specific inhibitor of S6K1 activation [13] (Figure 2). These data correlate with our published report for endogenous S6K1 activity [27].

Figure 3 Inhibition by EGTA of serum-stimulated S6K1 activity

HEK293 cells were transfected with wild-type S6K1 (2B4) or one of the mutants D3E, dE, dED3E and ΔC, and incubated with (ES) or without (S) 10 mM EGTA (20 min), followed by stimulation with 20% (v/v) FBS (20 min). Cells were harvested as described in the Experimental section. Myc-tagged S6K1 protein was immunoprecipitated and activity determined. (A) Typical experiment comparing the activities of each mutant; values are means $\pm$ S.E.M. (B) Activity of each mutant in the presence of EGTA expressed as a percentage of that following serum stimulation alone. The results from four separate experiments are combined and plotted as means $\pm$ S.E.M. The significance of the observed differences in enzyme activity was determined using a paired Student's *t* test: **P* < 0.05; ***P* < 0.005; ****P* < 0.0005; n.s., not significant.

Figure 4 Inhibition by EGTA of basal S6K1 activity

HEK293 cells were transfected with wild-type S6K1 (2B4) or with one of the mutants D3E, dE, dED3E and ΔC, serum starved for 24 h and harvested with (E) or without (SS) treatment with 10 mM EGTA, as described in the Experimental section. Myc-tagged S6K1 proteins were immunoprecipitated and activity was determined. Activities are plotted as means $\pm$ S.E.M. of four experiments. The significance of the observed differences in enzyme activity was determined using a paired Student's *t* test: **P* < 0.05; ***P* < 0.005; ****P* < 0.0005; n.s., not significant.

Structure/function analysis of the calcium dependence of S6K1 activation

To investigate the calcium dependence of S6K1 activation, we used a number of S6K1 structure/function mutants that have been used previously to investigate the mechanism of S6K1 activation [14,30,33]. dE, D3E and dED3E all have specific threonine or serine residues mutated to a glutamic acid or aspartic acid respectively, to mimic phosphorylation at these sites [14]. dE has the Thr-389 site mutated to glutamic acid. D3E has Ser-411, Ser-418 and Ser-424 mutated to aspartic acid and Thr-421 mutated to glutamic acid, increasing basal activity [14]. The combination mutant, dED3E, also exhibits constitutive activity [14]. The ΔC mutant has the region comprising amino acids 398–502 deleted, which includes the auto-inhibitory domain phosphorylation sites; however, it is still stimulated strongly by growth factors [34].

Firstly we determined whether EGTA pretreatment altered serum stimulation of the activity of these structure/function mutants (Figure 3). Figure 3(A) shows the results of a typical experiment, indicating the relative S6K1 activity levels. Figure 3(B) shows the relative effects of EGTA pretreatment on serum-stimulated activity for each mutant as an average of four separate experiments. Serum stimulation of wild-type S6K1 (2B4) was reduced by approx. 50% by EGTA pretreatment. Similarly, the acidic mutant constructs of the step 2 (D3E) and step 3 (dE) phosphorylation sites were also calcium sensitive, indicating that, individually, these steps are not calcium regulated. Conversely, the combination mutant dED3E was resistant to calcium chelation, suggesting that phosphorylation at both steps 2 and 3 is required to render the kinase calcium-independent. In addition, the ΔC mutant was resistant to EGTA treatment, indicating that the calcium-dependent activation of the kinase may be conveyed via the deleted C-terminal sequence postulated to be involved in the priming of the enzyme for activation (step 1, Figure 1).

Consistent with previous reports, the various S6K1 mutants all showed increased basal levels of kinase activity (Figure 4).

Table 1 EGTA decreases the phosphorylation of wild-type S6K1, but not of the dED3E mutant

HEK293 cells were transfected with wild-type (2B4) or mutant dED3E S6K1, ^{32}P -labelled and serum starved, and then treated with serum or serum plus EGTA. ^{32}P -labelled S6K1 was immunoprecipitated, separated by SDS/PAGE and the incorporated label was quantified by autoradiography and densitometry. The results from four separate experiments are expressed as a percentage of phosphorylation following serum stimulation, and are means $\pm$ S.E.M. The significance of the observed differences in enzyme activity was determined using a paired Student's *t* test: **P* < 0.05; ***P* < 0.005.

S6K1	Phosphorylation (%)		
	Serum-starved	Serum alone	EGTA + serum
Wild type	71.2 $\pm$ 6.5*	100	46 $\pm$ 10.3**
dED3E	—	100	93 $\pm$ 14

As for serum-stimulated activity, in the absence of growth factors the activity of wild-type and the step 2 (D3E) and step 3 (dE) mutants was sensitive to calcium chelation. The combination mutant was independent of calcium levels, while the ΔC mutant was resistant to calcium chelation (Figure 4). Thus

the combined activation of steps 2 and 3, or the release of the N-terminus–C-terminus interaction (step 1), is required for both serum-stimulated and basal S6K1 activity.

Based on these observations, we propose that the calcium-dependent disruption of an inhibitory interaction between the N- and C-termini is required for the activation of S6K1, and that this process is mimicked by deletion of the C-terminal 104 residues. This enables sequential phosphorylation of the step 2 and step 3 sites by calcium-independent kinases (mimicked by the dED3E mutation) before final activation by the PDK1-catalysed phosphorylation of Thr-229. This is consistent with our previous finding that the combined acidic mutant (dED3E) was synergistically phosphorylated by PDK1 when compared with the individual mutants (dE and D3E) [30]. The resistance of this mutant to calcium chelation indicates that step 4 is calcium independent, a fact reinforced by our previous finding that activation of the related PDK1-dependent kinase Akt is independent of calcium levels [27].

Calcium regulation of S6K1 phosphorylation

The results presented above indicate that the calcium-dependent signal crucial for S6K1 activation does not target the major

Figure 5 Tryptic phosphopeptide two-dimensional mapping reveals no phosphopeptide-specific effects of calcium chelation

HEK293 cells were transfected with wild-type S6K1 (2B4), ^{32}P -labelled and treated with serum (A, C) or serum following pretreatment with EGTA (B, D). ^{32}P -labelled S6K1 was immunoprecipitated, digested with trypsin, and then the peptides were extracted and either equal amounts of radioactivity (A, B) or equal protein equivalents (C, D) were separated by TLC as described in the Experimental section. Radiolabelled phosphopeptides were visualized after a 24 h exposure using a PhosphorImager and ImageQuant software (Molecular Dynamics). This figure is representative of four separate experiments, and panel (A) shows the location of known phosphorylation sites.

Figure 6 HPLC/TLC mapping reveals no phosphopeptide-specific effects of calcium

HEK293 cells were transfected with wild-type S6K1 (2B4), ^{32}P -labelled and treated with serum (A), serum plus EGTA (B) or ionomycin (C). ^{32}P -labelled S6K1 was immunoprecipitated and digested with trypsin, then the peptides were extracted and equal amounts of radioactivity were separated by HPLC. After HPLC, an equal volume of each fraction was separated by TLC as described in the Experimental section. Radiolabelled phosphopeptides were visualized after a 24 h exposure using a PhosphorImager and ImageQuant software (Molecular Dynamics). The figure is representative of three separate experiments. FT, flow through.

regulatory phosphorylation sites directly. However, given the complex and essential role played by phosphorylation in the regulation of S6K1, it is still possible that calcium-dependent regulation is achieved via phosphorylation of alternative sites. For example, sites within the N- or C-terminal regions that are involved in the priming step of S6K1 activation may be calcium sensitive. To address this possibility, HEK293 cells expressing either wild-type S6K1 or the dED3E EGTA-resistant mutant were metabolically labelled with ^{32}P and stimulated with serum in the absence or presence of EGTA. Overall, phosphorylation of S6K1 was significantly increased (29.2%; $P < 0.05$) by serum stimulation of quiescent HEK293 cells expressing wild-type S6K1 (Table 1). Moreover, S6K1 phosphorylation was significantly reduced (to 46%; $P < 0.005$) by EGTA pretreatment, whereas dED3E labelling was unaffected (Table 1). These results correlated well with those observed above measuring S6K1 activity (Figure 2).

We extended this study to determine if the reduction in phosphorylation was attributed to effects on specific phosphorylation sites, or to an overall decrease at all sites. Two-dimensional tryptic phosphopeptide mapping of equal amounts of radioactivity of ^{32}P -labelled wild-type S6K1 from cells treated with serum (Figure 5A) or pretreated with serum and EGTA (Figure 5B) revealed no significant phosphopeptide-specific effects of calcium chelation. Similarly, when equal protein equivalents were loaded in a separate mapping experiment, a decrease in phosphorylation of all peptides was observed with EGTA pretreatment, consistent with the effects on total S6K1 phosphorylation (Figures 5C and 5D).

To examine the pattern of phosphorylation more closely, the above tryptic phosphopeptides from cells treated with serum (Figure 6A), serum plus EGTA (Figure 6B), or ionomycin

(Figure 6C) were separated by HPLC and then by TLC. In all cases, equal counts were loaded on the HPLC, and the same volume of each fraction was spotted on the TLC to best facilitate comparison of the relative phosphorylation levels. A number of phosphorylated peptides were observed; however, there were no significant differences in the profiles for the three samples.

Investigation of a possible calcium-dependent binding protein interacting with S6K1

Taken together, these data indicate that calcium chelation results in a global decrease in S6K1 phosphorylation, consistent with the structure/function analysis. These results suggest the requirement for a phosphorylation-independent, calcium-dependent priming step before the subsequent activating sequence of phosphorylation reactions. In light of these data, we hypothesized that this priming event is dependent on a calcium-regulated binding protein. Based on experiments utilizing transient co-expression of S6K1 with candidate proteins, the small GTPase Cdc42/Rac1 [35], PKC ζ and PDK1 [31,36] have been shown to interact with S6K1. Romanelli et al. [31] went further to suggest the existence of a multimeric S6K1 signalling complex which includes PKC ζ and PDK1. Balendran et al. [36] also reported that formation of the PDK1–S6K1 complex was mediated via the hydrophobic motif surrounding Thr-389 of S6K1. We have confirmed the interaction of PDK1 and S6K1 in HEK293 cells co-transfected with both kinases; however, this association was unaffected by pretreatment with EGTA (results not shown), consistent with our published data [27] indicating that PDK1 is unlikely to play a role in the calcium-dependent regulation of S6K1.

The above studies were all performed utilizing protein over-expression. In order to determine whether the activation of S6K1 involves the formation of an endogenous calcium-dependent signalling complex, extracts from HEK293 cells that had been serum starved, stimulated with serum or stimulated with serum before or after treatment with EGTA, or following pretreatment with the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin, were chromatographed on a Superdex 200 gel filtration column. Fractions were separated on SDS/10%-PAGE and subjected to Western blot analysis with anti-S6K1 antibody (Figure 7A). Indeed, serum stimulation resulted in the assembly of S6K1 into a high-molecular-mass complex of > 440 kDa (Figure 7A; compare the first two panels). However, pretreatment with EGTA (Figure 7A, panel 3) or wortmannin (panel 4) markedly reduced the level of kinase in this complex, while increasing the proportion of monomeric S6K1. This effect was not simply an indication of elevated levels of inactive S6K1, as a similar large complex was observed in samples pretreated with rapamycin, a potent inhibitor of S6K1 signalling (results not shown). Furthermore, treatment of serum-stimulated HEK293 cells with 10 mM EGTA also decreased complex formation (Figure 7A, panel 5), indicating that calcium was required for the stability of the complex as well as for its formation. In these experiments, chromatography was performed in minimal buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 50 mM NaF, 40 mM β -glycerophosphate, 0.1 mM sodium vanadate, 1 mM benzamidine and 0.5 mM PMSF). Consistent with a role for calcium in complex stability, addition of 10 mM EGTA to serum-stimulated extracts and chromatography buffer prior to separation also decreased the level of S6K1 in the high-molecular-mass complex (results not shown).

Taken together, these data suggest for the first time the presence of a calcium-dependent binding protein interacting with S6K1 that is required to release the inhibition due to the interaction between the N- and C-termini (step 1 of S6K1

Figure 7 S6K1 exists in a high-molecular-mass complex that is sensitive to calcium chelation

(A) HEK293 cells were serum starved (top panel) and then stimulated with serum alone (panel 2), or stimulated with serum after treatment with EGTA (panel 3) or wortmannin (panel 4). In addition, serum-stimulated cells were subsequently treated with EGTA (panel 5). Cells were harvested in lysis buffer containing 0.1% NP40, as described in the Experimental section. Equal amounts of protein were chromatographed on a Superdex 200 gel filtration column in buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 50 mM NaF, 40 mM β -glycerophosphate, 0.1 mM sodium vanadate, 1 mM benzamide and 0.5 mM PMSF. The resulting fractions were separated on SDS/PAGE, transferred to Immobilon-P and probed with antibodies recognizing S6K1. (B) Superdex 200 gel filtration fractions of extracts from serum-stimulated cells were separated on SDS/PAGE, transferred to Immobilon-P and probed with antibodies recognizing PKC ζ , PKC α and Rac1. (C) Alternatively, fractions 2–5, 7–10 and 12–15 were pooled and PDK1 was immunoprecipitated. The complex was separated on SDS/PAGE, transferred to Immobilon-P and probed with anti-PDK1 antibody as described in the Experimental section. +ve Ctl, positive control. The Figures are representative Western blots of three separate experiments.

activation). Importantly, formation of the resultant high-molecular-mass complex is regulated by growth factors in a PI3K-dependent fashion. In an attempt to identify members of this complex, the gel filtration fractions from serum-stimulated cells were analysed for the presence of the previously reported interacting proteins. Western blot analysis revealed that PKC ζ exists in a complex of lower molecular mass than the S6K1 complex, while Rac1 was entirely in the monomeric form (Figure 7B). We have proposed previously that the conventional PKC isoforms are likely candidates to mediate the calcium-dependent activation of S6K1, at least in part [27]. PKC α is the predominant conventional PKC isoform in HEK293 cells (results not shown); however, Western blot analysis revealed that this isoform is absent from the high-molecular-mass complex containing S6K1 (Figure 7B, middle panel).

Initially, endogenous PDK1 was not detected in these analyses due to its low abundance. To increase the sensitivity of this analysis, fractions containing the high-molecular-mass (fractions 2–5) or monomeric (fractions 7–10) forms of S6K1, and those containing no detectable S6K1 (fractions 12–15), were combined, and PDK1 was immunoprecipitated and detected by Western blot analysis. PDK1 was only immunoprecipitated in fractions 7–10, consistent with its monomeric form (70 kDa), and was not detected in high-molecular-mass fractions 2–5 (Figure 7C). This observation is consistent with our biochemical data indicating that PDK1 activity is not responsible for the calcium dependence of S6K1 activity.

DISCUSSION

S6K1 is a member of the AGC family of kinases, which includes PKC, PKA, Akt and SGK. All of the AGC family members contain regulatory phosphorylation sites analogous to Thr-229 and Thr-389 in S6K1, and hence share related mechanisms of activation, including activation loop phosphorylation by PDK1 [20,22–26]. Despite these conserved regulatory mechanisms, each appears to have adapted to allow these enzymes to perform very distinct cellular roles [37]. We have shown previously that a major difference in the signalling between Akt and S6K1 is the calcium requirement of S6K1 [27], and have used this observation in the present study to identify the step required for the independent regulation of S6K1 activation.

The activation of S6K1 is a complex process involving both structural changes and phosphorylation of a number of key sites. However, despite extensive research for over 10 years, a number of basic aspects of the mechanism by which S6K1 is activated remain to be elucidated. For example, there is still no direct indication of how the activating phosphorylation cascade is initiated. Moreover, the kinases responsible for phosphorylating the key auto-inhibitory and hydrophobic motif sites remain to be identified definitively.

Using structure/function mutants and *in vivo* metabolic labelling, we have shown that the calcium activation pathway does not target a specific phosphorylation site, and that this dependence is only circumvented by deletion of the C-terminal 104 residues or by combining activating mutations of steps 2 and 3 (see Figure 1). Furthermore, we have demonstrated that the step 4 kinase PDK1 is independent of calcium both for its activity and for its ability to interact with S6K1. Based on these findings, we propose that calcium regulates the initiating step of S6K1 activation, involving disruption of an inhibitory interaction between the N- and C-termini. This hypothesis is consistent with previous findings indicating that this interaction regulates S6K1 activity [15,30], and our previous proposal that phosphorylation of Thr-229 (step 4) is regulated via an intrasteric mechanism [30].

The role of signalling complexes in the activation of a range of protein kinases is being increasingly recognized. Complexes involving JNK (c-Jun N-terminal kinase) [38] and PKA have been studied extensively [39,40]. Indeed, an S6K1 signalling complex has been proposed to include PDK1 and PKC ζ [31]. To date, the majority of studies have used co-immunoprecipitation of overexpressed candidate S6K1 partners as a measure of interaction [31,35,41]. Here we have shown, for the first time, the existence of an *in vivo* high-molecular-mass S6K1 complex containing endogenous proteins. Furthermore, the formation and stability of this complex is dependent on the maintenance of intracellular calcium levels. We propose that a calcium-dependent binding protein is required for complex formation, facilitating activation of the enzyme. Romanelli et al. [31] have proposed that S6K1 exists in a PI3K-regulated signalling complex; however, they failed to demonstrate any growth factor dependence of the complex formed upon co-transfection of S6K1, PKC ζ and PDK1. In our present study, we found that the formation of endogenous high-molecular-mass complexes containing S6K1 is dependent on both growth factors and PI3K, consistent with a functional role in the regulation of S6K1. Indeed, the step 2/step 3 acidic mutant dED3E is resistant to both calcium chelation (the present study) and wortmannin treatment [15], consistent with the calcium- and PI3K-dependent activation of S6K1 being mediated, at least in part, via formation of the high-molecular-mass signalling complex.

This endogenous signalling complex does not appear to contain PDK1, PKC ζ , PKC α or Rac1, molecules identified as potential interacting partners of S6K1 in transient transfection assays. However, our analysis does not preclude a functional role for low-stoichiometry complexes that are not detected in this system. Analysis of the composition of the S6K1-containing high-molecular-mass complex will provide important clues for understanding the regulation of this key enzyme. In light of the essential role that S6K1 plays in protein synthesis and thus cellular growth, understanding its regulation will have major implications not only for the control of normal growth but also in cases of abnormal growth such as cancer.

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