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A phospho-proteomic screen identifies novel S6K1 and mTORC1 substrates revealing additional complexity in the signaling network regulating cell growth

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ABSTRACT

S6K1, a critical downstream substrate of mTORC1, has been implicated in regulating protein synthesis and a variety of processes that impinge upon cell growth and proliferation. While the role of the cytoplasmic $p70^{S6K1}$ isoform in the regulation of translation has been intensively studied, the targets and function of the nuclear $p85^{S6K1}$ isoform remain unclear. Therefore, we carried out a phospho-proteomic screen to identify novel $p85^{S6K1}$ substrates. Four novel putative $p85^{S6K1}$ substrates, GRP75, CCTβ, PGK1 and RACK1, and two mTORC1 substrates, ANXA4 and PSMA6 were identified, with diverse roles in chaperone function, ribosome maturation, metabolism, vesicle trafficking and the proteasome, respectively. The chaperonin subunit CCTβ was further investigated and the site of phosphorylation mapped to serine 260, a site located in the chaperonin apical domain. Consistent with this domain being involved in folding substrate interactions, we found that phosphorylation of serine 260 modulates chaperonin folding activity.

1. Introduction

Cellular growth is a prerequisite for timely cell division and is tightly regulated in order to achieve optimal cell size under specific environmental conditions. A key pathway which integrates both growth factor and nutritional cues to regulate cell growth and proliferation is the PI3K/mTOR signaling pathway [1]. While mammalian cells express only one mTOR protein, it assembles into two complexes, mTOR complex 1 and 2 (mTORC1/2), which have distinct cellular functions. mTORC1 is a rapamycin sensitive complex composed of mTOR, Raptor, mLST8, PRAS40 and Deptor, while mTORC2 is rapamycin insensitive and composed of mTOR, Rictor, mSIN1, mLST8, Protor-1 and Deptor [1]. Although the latter complex was initially implicated in the regulation of the cytoskeleton [2,3], more recent studies have identified it as an upstream regulator of AKT and SGK [4–7], demonstrating that mTORC2 can also regulate cell survival, metabolism and proliferation via these substrates.

The central role ascribed to mTORC1 is regulation of cellular growth with two of its best characterized substrates, initiation factor 4E-binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase 1 (S6K1) playing critical roles in the regulation of protein synthesis both at the level of efficiency of the existing translation machinery, as well as through regulation of the rates of ribosome biogenesis [8]. mTORC1-mediated phosphorylation of 4E-BP1 relieves its inhibition on the eukaryotic initiation factor 4E (eIF4E), allowing the latter to bind the mRNA 5′ cap and recruit additional factors to initiate mRNA translation [9,10]. Similarly, the phosphorylation and thus activation of S6K1 by mTORC1 promotes a complex cascade of downstream signaling to substrates involved in the regulation of protein translation, such as ribosomal protein S6, eIF4B, PDCD4 and eEF2K [11–15]. In addition, S6K1 has also been implicated in the regulation of mRNA splicing via the phosphorylation of SKAR [16], as well as regulation of cell survival via the phosphorylation of BAD and MDM2 [17,18]. Importantly, S6K1 also mediates feedback inhibition of the PI3K/AKT pathway under conditions of chronic insulin stimulation, or loss of the TSC1/2 complex, through the phosphorylation of IRS1, leading to its degradation [19,20]. Most recently, a complementary negative feedback loop has been identified whereby S6K1 phosphorylates Rictor, resulting in a reduction of mTORC2-mediated AKT activation [21–23]. Furthermore, S6K1 also phosphorylates mTOR directly and although the functional significance of this phosphorylation is unknown, it possibly acts in a positive feedback manner [24].

Despite extensive studies to date, the complexity of S6K1 signaling is still not fully understood. For example, we have identified a nuclear

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role for S6K1 in the regulation of ribosomal RNA gene (rDNA) transcription, although the direct S6K1 substrate(s) involved in this process are as yet unknown [25]. Thus in this study we hypothesized that additional nuclear S6K1 substrates exist, allowing it to regulate and coordinate functions required for cellular growth and proliferation.

To this end, we carried out a screen to identify novel nuclear proteins phosphorylated by the nuclear localized form of S6K, $p85^{S6K1}$, using 2 dimensional-gel electrophoresis (2D-GE) and immunoblotting for proteins phosphorylated within the S6K1 consensus motif. Using this approach we identified four novel S6K1 substrates (GRP75, CCTβ, PGK1 and RACK1) and two mTORC1 regulated substrates (ANXA4 and PSMA6) involved in regulating chaperone, ribosome maturation, metabolism, vesicle trafficking and proteasomal functions. One of the S6K1 substrates identified, CCTβ, was further characterized and the site of phosphorylation mapped to S260. We demonstrate that this site is directly phosphorylated by S6K1 *in vitro* and in intact cells in a rapamycin-sensitive manner, indicating that CCTβ is a *bona fide* novel S6K1 substrate. Furthermore, we show that mutation of the S260 site does not affect CCTβ assembly into the chaperonin holocomplex but is required for its substrate folding activity. Thus our studies implicate the S6K1/mTOR pathway in the regulation of a number of cellular, and more specifically nuclear, functions previously not associated with this pathway.

2. Materials and methods

2.1. Expression constructs

pRK5-myc-$p70^{S6K1}$-dED_3E construct has been described previously [25–27]. The equivalent mutant was generated by subcloning the C-terminal region of the above construct into pRK5-myc-$p85^{S6K1}$ (a kind gift from G. Thomas, Genome Research Institute, Cincinnati). The myc-$p70^{S6K1}$-dED_3E and myc-$p85^{S6K1}$-dED_3E ORFs were subcloned into the MSCV-IRES-GFP vector (a kind gift from S. Russell, Peter MacCallum Cancer Centre). The ORF from pET11d-CCTβ (a kind gift from K.R. Willison, Institute of Cancer Research, UK) was N-terminally His- or Flag-tagged and subcloned into pET-17b (Novagen) or MSCV-IRES-GFP, respectively. The S260 site was mutated to alanine (A) or aspartic acid (D) by site-directed mutagenesis.

2.2. Cell lines and culture conditions

NIH 3T3, HEK 293 and HEK 293T cells were cultured in DMEM with 10% FBS and 1% antibiotic–antimycotic (Invitrogen) in 5% CO_2 atmosphere at 37 °C. NIH 3T3 stable cell lines expressing S6K1 or CCTβ were generated using standard methods for transduction with retroviral MSCV-IRES-GFP supernatants and selection using FACS. Where indicated, cells were serum starved by washing once with PBS and incubation in DMEM containing 0.5% BSA, 1% antibiotic–antimycotic for 24 h. Stimulation with 10% FBS was performed for 30 min or 3 h, with rapamycin (20 nM) pre-treatment for 30 min or 1 h, as indicated.

2.3. 2D-GE

Nuclear enriched protein lysates were prepared using the NucBuster Protein Extraction Kit (Novagen) as per the manufacturer's instructions. Protein (800 μg/gel) was precipitated with acetone, resuspended in 300 μl of focusing buffer (8 M urea, 2% CHAPS, 1% tributylphosphine (Bio-Rad) 0.5% Bio-Lyte 3/10 Ampholyte (Bio-Rad)) and used to rehydrate ReadyStrip IPG 3–10 strips (17 cm; Bio-Rad). Proteins were separated using the Protean IEF System (Bio-Rad) as per the manufacturer's instructions. Thiol groups were reduced and alkylated by incubation in 2% (w/v) DTT, followed by 2.5% (w/v) iodoacetamide, both prepared in equilibration buffer (6 M urea, 375 mM Tris–HCl, pH 8.8, 2% SDS, 20% glycerol). Proteins were separated alongside Benchmark Pre-Stained Protein Ladder (Invitrogen) by SDS-PAGE run at constant amperes at 10 °C. Duplicate gels were run, one was transferred onto PVDF and immunoblotted using the Phospho-AKT Substrate antibody (Cell Signaling) and the other stained with Coomassie Blue R-250 (ICN Biomedicals).

2.4. Identification of proteins by mass spectrometry

Proteins were excised and in-gel trypsin digested (sequencing Grade Modified Trypsin (Promega) in 50 mM NH_4HCO_3, 10% (v/v) CH_3CN) overnight at 37 °C. Supernatants were collected, peptides extracted with TFA and CH_3CN, concentrated and resuspended in 2% (v/v) TFA. Tandem mass spectrometry was carried out on an LC-ESI/Ion Trap Mass Spectrometer coupled to a HPLC-Chip (Agilent) at the Bio 21 Institute Visitor's Lab (Melbourne, Australia). Peptide fragment data were acquired using MSD Trap Control Software and exported in MASCOT Generic File (MGF) format. Data were analyzed using MASCOT software (Matrix Science) against the NCBInr database, setting peptide mass tolerance at ± 2 Da. A Mowse score of greater than 50 for four or more peptides was deemed as positive identification.

2.5. Expression of His-CCTβ

pET-17b-His-CCTβ was transformed into BL21(DE3)pLysS cells and expressed using the Overnight Express Autoinduction System 1 (Novagen). Cells were sonicated (Branson Sonifier Cell Disruptor B15), the insoluble fraction collected and resuspended in urea buffer (8 M urea, 300 mM NaCl, 29 mM Na_2HPO_4, 21 mM NaH_2PO_4, pH 7). The sample was incubated with Talon resin (Clontech) then washed with urea buffer and protein eluted using 150 mM imidazole. Fractions containing His-CCTβ were dialysed using Membra-Cel MD10-14 (Viskase) against decreasing urea concentrations, into a final buffer of PBS, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was determined by D_c Protein Assay (Bio-Rad).

2.6. Phosphorylation of CCTβ in vitro

HEK 293T cells were calcium phosphate transfected with pRK5-myc-$p70^{S6K1}dED_3E$ and pRK5-myc-$p85^{S6K1}dED_3E$ constructs. Cells were lysed in 50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 40 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM benzamidine, 1% (v/v) NP-40, 0.5 mM PMSF and protein concentration determined as above. $p70^{S6K1}$ or $p85^{S6K1}dED_3E$ were immunoprecipitated using a myc-epitope tag (9E10) antibody [26,27] and incubated with GST-rpS6 peptide [27] or His-CCTβ in the presence of ^{32}P-ATP.

2.7. Phosphopeptide mapping, phospho-amino acid analysis and phosphate release analysis

In vitro phosphorylated CCTβ was resolved by SDS-PAGE, Coomassie stained and excised from gel. Proteins were in-gel trypsin digested, separated by Thin Layer Electrophoresis, then Thin Layer Chromatography (TLC) [25,27]. The plate was air-dried, exposed to a phosphoimager screen and analyzed using the Storm 820 Phosphoimager and ImageQuant software (GE Healthcare). To carry out phospho-amino acid analysis, ^{32}P-labeled phosphopeptides were recovered, hydrolyzed, spotted onto a TLC plate, separated by electrophoresis, and stained with ninhydrin as described previously [28]. The plate was exposed to a phosphoimager screen and analyzed as above. The phosphorylated amino acid was determined by comparison to standards. For phosphate release analysis, the major ^{32}P-labeled phosphopeptide was eluted