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S6 Kinase 2 Is Bound to Chromatin-Nuclear Matrix Cellular Fractions and Is Able to Phosphorylate Histone H3 at Threonine 45 In Vitro and In Vivo

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ABSTRACT

The activity of S6 kinases (S6K) is highly induced in cancer cells highlighting an essential role in carcinogenesis. The S6K family has two members: S6K1 and S6K2 which bear common as well as distinct features. In an attempt to identify S6K2 unique sequence features compared to S6K1, we applied extensive bioinformatic analysis and motif search approaches. Interestingly, we identified 14 unique protein signatures which are present in proteins directly connected to chromatin and/or involved in transcription regulation. Using chromatin binding assay, we biochemically showed that S6K2 is bound to chromatin as well as nuclear matrix cellular fractions in HEK293 cells. The presence of S6K2 in chromatin fractions raised the possibility that it may be in close proximity to a number of chromatin substrates. For that, we then searched for S6K phosphorylation consensus sites RXRXXT/S in mammalian proteins using the SWISS-PROT database. Interestingly, we identified some potential phosphorylation sites in histone H3 (Thr45). Using in vitro kinase assays and siRNA-based knockdown strategy; we confirmed that S6K2 but not S6K1 or AKT is essential for histone H3-Thr45 phosphorylation in HEK293 cells. Furthermore, we show that the nuclear localisation sequence in the S6K2 C-terminus is essential for this modification. We have found that, H3-Thr45 phosphorylation correlates to S6K activation in response to mitogens and TPA-induced cell differentiation of leukaemic cell lines U937, HL60 and THP1. Overall, we demonstrate that S6K2 is a novel kinase that can phosphorylate histone H3 at position Thr45, which may play a role during cell proliferation and/or differentiation. J. Cell. Biochem. 115: 1048–1062, 2014.

KEY WORDS: S6K2; H3 PHOSPHORYLATION; CHROMATIN

The 40S ribosomal protein S6 kinase (S6K) is a downstream target of mTORC1 [Jacinto and Lorberg, 2008]. S6K is represented by two homologous cellular proteins, S6K1 and S6K2, both of which act downstream of mTOR and phosphorylate S6 ribosomal proteins [Lee-Fruman et al., 1999]. Although there are two homologues of S6K [Koh et al., 1999; Lee-Fruman et al., 1999], most of the studies have been focused on S6K1 and little is known about the function of S6K2. S6K1-deficient mice showed persistent S6 phosphorylation but had a small body phenotype [Shima et al., 1998]. S6K1/2 double-knockout mice also exhibit normal proliferation and growth reduction [Pende et al., 2004]. Similarly, S6K1/2 double-knockout mouse embryo fibroblasts and myoblasts show defects in size but not proliferation [Pende et al., 2004; Ohanna et al., 2005; Dowling et al., 2010]. These results suggest that these two homologues have redundant as well as non-overlapping functions. It has been reported that S6K2 but not S6K1 was important for fibroblast growth factor 2 (FGF2)-induced chemoresistance of small cell lung cancer cells [Pardo et al., 2006]. A recent study showed that S6K2 but not S6K1 was important for cell proliferation in response to mTOR activation [Goh et al., 2010].

S6K2 has been mapped to the chromosome 11q13 locus; amplification at this locus is commonly observed in breast, ovarian, head and neck, oral and oesophageal cancer. Studies of this region led

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to the identification of multiple amplicons containing several potential oncogenes [Brown et al., 2008]. 11q13 amplification is a late-stage event in several cancers that is often associated with poor prognosis [Rodrigo et al., 2009]. Interestingly, nuclear staining of S6K2 increases in breast adenocarcinomas in comparison to normal tissue [Filonenko et al., 2004]. Furthermore, a specific role of S6K2 in promotion of cell survival in breast cancer was linked through AKT [Sridharan and Basu, 2011]. Those results hint at a distinct role of S6K2 than its relative kinase S6K1 in cancer. This makes it very useful to identify S6K2 specific substrates and functions in carcinogenesis.

A comparison of the S6K2 sequence with its homologue S6K1 revealed that they are highly similar (70% overall), sharing 82% identity in the catalytic domain [Gout et al., 1998; Shima et al., 1998; Lee-Fruman et al., 1999]. Moreover, all of the important regulatory domains are conserved, including the acidic N-terminus, the linker region and the autoinhibitory domain. Remarkably, the C-termini of both S6K isoforms exhibit only 20% identity. The S6K1 C-terminus sequence contains a PDZ domain binding motif which might recruit the kinase to the actin cytoskeleton via binding to neurabin [Burnett et al., 1998]. In S6K2, a number of distinct features reside in its C-terminal regulatory region, including nuclear localisation sequence (NLS), proline-rich motif and several regulatory phosphorylation sites. The presence of functional nuclear localisation motif at the C-terminus has been demonstrated by Lee-Fruman et al. [1999]. Phosphorylation of S6K2 (p54) by protein kinase C (PKC) at S486 in the C-terminal region was shown to disable the function of the NLS, resulting in the accumulation of phosphorylated S6K2 in the cytoplasm [Valovka et al., 2003]. Recently, S6K2 but not S6K1 was reported to be localised at centrosomes [Rossi et al., 2007]. However, the physiological relevance of centrosomal localisation of S6K2 remains to be further investigated. The presence of a stretch of proline-rich sequences implicates the C-terminus of S6K2 in mediating protein–protein interactions with SH3 domain-containing proteins [Gout et al., 1998; Lee-Fruman et al., 1999].

Alternative splicing in S6K2 genes results in two isoforms which are both predominantly nuclear. Only recently, S6K2 was linked to nuclear substrates as the acetyltransferase p300 [Fenton et al., 2010] and associates with a number of RNA-binding proteins, including heterogeneous ribonucleoproteins (hnRNPs) [Goh et al., 2010] through a direct physical interaction. Recently, we identified an additional link for S6K2 to the nucleus compartment through the direct interaction with the general transcription factor Yin Yang 1 (YY1) (Ismail et al., 2013).

In the present study, we extended the analysis of S6K2 amino acid sequence in comparison to S6K1 by using motif search bioinformatic approaches. We identified 14 unique protein signatures exclusive to S6K2. For instance, we have found that S6K2 sequence contains signatures homologous to homeobox domain, HOX9 activation region signatures, protein signature of regulation of prenuclear mRNA protein all of them resides in the extreme C-terminus and three signatures of the high mobility group proteins (HMG) in the N- and C-termini. Members of the superfamily of high mobility group (HMG) proteins are considered as architectural elements of chromatin. It is now clear that they belong to a network of dynamic chromatin proteins that constantly move around the chromatin fibre thereby dynamically modulating DNA-dependent processes [Catez and Hock, 2010].

The preferred S6K phosphorylation motif RXRXXS/T (where X is any amino acid) was found in most of the S6K substrates identified to date. Using bioinformatic analysis, we identified a number of nuclear proteins involved in gene transcription regulation including histone H3-Thr45 that contain a sequence conforms to S6K substrate recognition site. Histone H3 threonine 45 phosphorylation was initially identified by Hurd et al. [2009], who found that H3-Thr45 phosphorylation increases dramatically in apoptotic neutrophils. Additionally they identified PKC-delta as the kinase responsible for H3-Thr45 phosphorylation in vitro and in vivo. In *Saccharomyces cerevisiae*, Baker et al. [2010] reported the phosphorylation of H3-Thr45 within the H3 alphaN helix. They found that Thr45 phosphorylation peaks during DNA replication, and is mediated by the S phase kinase Cdc7-Dbf4 as part of a multiprotein complex. Furthermore, the loss of phosphorylated H3-Thr45 caused phenotypes consistent with replicative defects, and prolonged replication stress resulted in H3-Thr45 phosphorylation accumulation over time [Baker et al., 2010]. A more recent study by Villagrasa et al. [2012] identified AKT2 as a kinase that phosphorylate H3 at Thr45 after binding to Snail1 transcription factor. This interaction was observed in the nucleus and increased the intrinsic Akt2 activity on histone 3 Thr45. Phosphorylation of Thr45 in histone H3 is sensitive to Snail1 and Akt2 cellular levels; moreover, Snail1 upregulates the binding of phosphoThr45 histone H3 to the E-cadherin (CDH1) promoter [Villagrasa et al., 2012].

In the present study, we identified S6 kinase 2 as a novel kinase that is able to use histone H3 as a substrate and targets its phosphorylation at Thr45 residue. Here, we show the identification of a number of protein signatures in S6K2 sequences that are found in chromatin/DNA bound proteins. We biochemically validated the presence of S6K2 in chromatin and nuclear matrix cellular fractions. We report that S6K2 but not S6K1 or AKT is able to phosphorylate Histone H3 at Thr45 in vitro and in HEK293 cells. Interestingly, the disruption of S6K2 nuclear localisation sequence significantly affects H3-Thr45 phosphorylation. Furthermore, we show that H3-Thr45 phosphorylation is linked to S6K activation in response to cellular stimuli including mitogens and TPA-induced cell differentiation.

MATERIALS AND METHODS

PLASMIDS, RECOMBINANT PROTEINS AND ANTIBODIES

The Glu-Glu (EE-tagged) expression constructs for S6K1 and S6K2 were constructed as previously described [Valovka et al., 2003]. S6K2 nuclear localisation mutant (S6K2-NLS) was constructed from S6K2 wild-type vector using an antisense primer incorporating the desired mutation and an *Eco*RI restriction site at the 3′-end of S6K2 gene (5′-CGCGAATTCTCAGGACTTCTTGGTCCCTGATTT-3′). This was used together with the standard S6K2 sense primer which contains a *Bam* HI site as described previously [Valovka et al., 2003] for PCR and the resulting fragment was cloned into pcDNA 3.1. His-tagged S6K1 and 2 were purified from Sf9 insect cells as described previously [Valovka et al., 2003]. 80S ribosomes were purified from rat liver as described previously [Thomas et al., 1978]. His- tagged histones and calf thymus histones were described previously [Hurd et al., 2009]. The monoclonal antibody to the EE tag, anti-S6K1 and anti-S6K2 rabbit polyclonal antibodies were described previously [Valovka

et al., 2003]. AKT antibody was from Santa Cruz Biotechnology. Phospho-rpS6 (S240/244) and phospho-S6K (T412) antibodies were from Cell Signaling Technology. Actin monoclonal antibody was from Sigma-Aldrich. Phospho-Thr45 (H3-Thr45) specific antibody was from Active motif and Abcam.

ESTABLISHING TETRACYCLINE INDUCIBLE STABLE CELL LINES

Stable cell lines were generated following the method we described recently(Ismail et al., 2013). Briefly, tetracycline-Regulated Expression System for mammalian cells (T-Rex System, Invitrogen) was used for this purpose. T-Rex HEK293 cells were cultured in the presence of 5 μg/ml blasticidin. EE-S6K2 and EE-S6K2- NLS mutant genes were cloned into pcDNA4/TO(+) vector and transfected in T-REX HEK293 for 48 hours. Cells were then split 1/20 into selective complete medium containing 5 μg/ml of blasticidin and 100 μg/ml of zeocine. Cells were allowed to grow over a period of two weeks. For each gene 20 clones were selected and screened for tetracycline-regulated protein expression. To induce protein expression tetracycline was added into each dish to a final concentration of 1 μg/ml and cells were incubated for 24 hours at 37°C. Positive cell clones for expression were frozen in DMEM medium containing 50% FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen. Cells were then maintained in full DMEM medium in the presence of 1 μg/ml blasticidin.

CELL CULTURE

The human monocytic cells THP-1, U937 and HL60 were cultured in RPMI 1640 medium. HEK293 and NIH3T3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM). Media were supplemented with 10% foetal bovine serum (Gibco BRL, Gaithersburg, MD) and 1 mg/ml penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2. *For cell differentiation*, THP-1, HL60 and U937 cells were diluted at 2×10^6 cells in 2 ml of RPMI and treated with 10 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which induces macrophagic terminal differentiation and cell adherence. Cell suspensions were then cultured on 6-well plates and monitored every 24 h to check for differentiation criteria using an inverted microscope. Adherent cells were then washed by PBS to remove non-adherent cells and then lysed as described below.

DNA AND SiRNA TRANSFECTIONS

For DNA transfection studies, cells were seeded at 5×10^6 cells per 6 cm plate and transfected using ExGen 500 (Fermentas Life Sciences) as recommended by the manufacturer. Forty-eight hours after transfection cells were harvested and lysed for analysis by Western blotting and immunoprecipitation. *SiRNA transfection*: Sequences of the siRNA duplexes used to knock down S6K1, S6K2, AKT and control siRNA are as follows: S6K1-sense 5′-GGACAUGGCAGGAGUGUUU-3′, S6K1-antisense 5′-AAACACUCCUGCCAUGUCC-3′, S6K2-sense 5′-GAACCAAGAAGUCCAAGAG-3′, S6K2-antisense 5′-CUCUUGGACUUCUUGGUCC-3′, AKT-sense 5′-UGCCCUUCUACAACCAGGA-3′, AKT-antisense 5′-UCCUGGUUGUAGAAGGGCA-3′, control-sense 5′-AGGUAGUGUAAUCGCCUUG-3′ and control-antisense 5′-CAAGGCGAUUACACUACCU-3′. A BLASTn search was carried out to detect possible off targets. The siRNA were manufactured and purified by HPLC by MWG Biotech. SiRNA duplexes were dissolved in sterile siMAX Universal buffer to give a concentration of 10 μM. For the knockdown of the selected proteins, HEK 293 cells were seeded at 0.5×10^6 cells per well in 6-well plate. The following day, 20 nM of siRNA was diluted in 250 μl of Opti-MEM®I reduced serum medium (Opti-MEM) (Invitrogen). Ten microlitres of Lipofectamine-2000 was diluted in a separate aliquot of Opti-MEM. After five minutes incubation at room temperature, the two solutions were combined and mixed gently. Mixtures were incubated for a further 20 min. During this time 2 ml of fresh medium containing no antibiotics was added to each plate of cells. The siRNA:Lipofectamine-2000 complexes were then added to each plate. Cells were incubated at 37°C for 72 h, then lysed to assay for gene knockdown efficiency by Western blotting.

IMMUNOPRECIPITATION AND IMMUNOBLOT ANALYSIS

Cells were lysed in EB-buffer (20 mM Tris–HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, EDTA-free protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation and equal amounts of total protein were used for each immunoprecipitation in the presence of 2 μg of anti-EE anitibodies and 30 μl of 50% protein A sepharose suspension. Samples were incubated on a rotating wheel overnight at 4°C. The beads were washed three times with ice-cold EB buffer and then used directly in kinase assays or analysed by Western blotting using specific antibodies.

S6K IN VITRO KINASE ASSAY

Kinase assay was done as described previously (Ismail et al., 2013). Briefly, EE-tagged S6K2WT or NLS-mutant was immunoprecipitated from transfected HEK 293 cells using anti-EE antibodies. After washing, the immune complexes were resuspended in 25 μl of kinase assay buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 10 mM β-glycerophosphate, PKA inhibitor) supplemented with 100 μM ATP, 5 μCi of [γ-32P] ATP (Amersham) and 2 μg of 80S ribosomes or 1 μg histone H3 as a substrate. The reaction was carried out at 30°C for 30 min and terminated by the addition of 5× Laemmli sample buffer. Proteins were resolved by SDS–PAGE and detected using Coomassie stain. The following day ^{32}P incorporation into the ribosomal S6 protein or Histone H3 was assessed by the Fujifilm FLA-2000 phosphor-imaging system and visualised using Fujifilm MultiGauge software.

CHROMATIN BINDING ASSAY AND NUCLEAR FRACTIONATION

For chromatin binding assay, the method of He et al., 1994 was used here, with modifications described by Kannouche et al., 2004. Briefly, 36 h after transfection cells were lysed for 15 min on ice in cold CSK I buffer (10 mM Pipes, (pH 6.8), 100 mM NaCl, 1 mM EDTA, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM DTT) supplemented with 0.5% (v/v) Triton X-100, protease inhibitors (Roche Complete Mini) and 1 mM phenylmethylsulfonyl fluoride. One-tenth of the lysate (total fraction, T) was mixed with RIPA buffer (150 mM Tris–HCl, (pH 8.0), 150 mM NaCl, 0.5% DOC, 0.1% (w/v) SDS, 1% (v/v) NP-40). The remaining cell lysate was divided into two equal portions, which were centrifuged at 500 ×g at 4°C for 3 min. The supernatants (S1 fraction), which contained Triton-soluble proteins, were further analyzed. One of the pellets, containing chromatin-bound, nuclear matrix-bound and insoluble proteins, was resuspended in RIPA buffer (the P1 fraction). The second was resuspended in CSK II buffer (10 mM Pipes (pH 6.8), 50 mM NaCl, 300 mM sucrose, 6 mM $MgCl_2$, 1 mM DTT), treated with DNase for 30 min followed by extraction with 250 mM $(NH_4)_2SO_4$ for

10 min at 25°C. The sample treated with DNase and salt was then centrifuged at 1200 ×g for 6 min at 4°C and the supernatant (S2 fraction, containing DNase-released chromatin-associated proteins) and pellet (P2, containing insoluble, cytoskeletal, and nuclear matrix proteins) were collected. P2 was also resuspended in RIPA buffer. All fractions were analyzed by immunoblotting. **Nuclear fractionation:** cultured cells were trypsinized and washed once with 1× phosphate-buffered solution, followed by resuspension in ice-cold hypotonic buffer (20 mm HEPES, 0.5 mm dithiothreitol, protease inhibitor mixture added, pH 7.9) for 15 min. 10% Nonidet P-40 was added after 15 min at a concentration of 40 μl/ml and vortexed for 10 s followed by centrifugation at 800 ×*g* for 1 min, 4°C. The resulting supernatant (cytoplasmic and cellular membrane fraction) was removed and saved separately on ice. The pelleted nuclei were washed once with hypotonic buffer supplemented with 240 μl/ml of 10% Nonidet P-40 with nuclei pelleted by centrifugation at 800 ×*g* for 1 min, 4°C. The nuclei were further washed and pelleted in hypotonic buffer alone. Ice-cold lysis buffer (20 mm Tris-HCl, pH 7.5, 1% Triton X-100, 150 mm NaCl, 5 mm EDTA, 50 mm NaF, protease inhibitor mixture) was added to lyse nuclear pellet for 30 min at 4°C, followed by centrifugation at top speed for 30 min at 4°C on a bench top centrifuge to remove nuclear debris.

FLOW CYTOMETRY ANALYSIS

Levels of phosphorylated H3 at Thr45 in the differentiated and undifferentiated THP-1 cells were determined by flow cytometry. Cells ($1–2 \times 10^6$) were washed 3 times with phosphate-buffered saline (PBS) and then fixed with 4% formaldehyde (methanol free) for 10 min at 37°C. After fixation cells were rinsed and then premabelised using 90% ice cold methanol for 30 min on ice. Cells were washed and blocked in 0.5% BSA for 10 min then incubated with anti- phospho Thr45 H3 antibody (1:1000) for 1 hr at room temperature. FITC-conjugated goat anti-Rabbit antibody was then added to cells for 30 minutes in dark. Cells were washed with PBS and then analyzed on a FACS Calibur flow cytometry. Data were processed using the Cell Quest software (Becton-Dickinson).

REAL-TIME REVERSE TRANSCRIPTION-PCR FOR CD36 GENE EXPRESSION

Relative mRNA levels of CD36 and GAPDH were quantified with real-time quantitative reverse transcription-PCR. Total RNA was extracted from undifferentiated and differentiated cells using RNeasy RNA purification kit (Qiagen, Valencia, CA). Total RNA was reverse transcribed using oligo-dT primers and the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA), according to the protocol of the manufacturer. PCR primers were obtained from MWG Biotech. The primer sequences for CD36 gene are forward: 5′-agtcactgcgacatgattaatggt-3′, reverse: 5′-ctgcaatacctggcttttctc-3′. GAPDH was used as an internal control for normalisation of samples and the primer sequences used are as following, forward: 5′-agggctgcttttaactctggt-3′ and reverse: 5′-ccccacttgattttggaggga-3′. The PCR reaction was performed using SYBER Green PCR Master mix (Applied Biosystems), and Qiagen Rotor Gene 6000 Real-Time PCR system, according to the instructions of the manufacturer. Reactions were performed in 20 μl total volume and incubated at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The results were analysed by Rotor gene 6000 Real-Time PCR System software.

STATISTICAL ANALYSIS

The results were analysed using GraphPad prism computer system (GraphPad Software, San Diego, USA). Statistical analysis comparisons were done with Student′s *t*-tests.

RESULTS

MOTIF SEQUENCE ANALYSIS OF S6K2 PROTEIN REVEALS THE PRESENCE OF CHROMATIN BOUND PROTEINS′ SIGNATURES

To identify S6K2 protein unique features, we inspected the S6K2 amino acid sequence compared to S6K1 using GenomeNet database MOTIF bioinformatics tools (http://www.genome.jp/tools/motif). We analysed the sequence of human S6K2 with accession number Q9UBS0 compared to human S6K1 sequence with accession number P23443. The analysis retrieved at least 190 motifs and the results were then filtered on basis of high score of homology with the original source sequence and whether the sequence is exclusive to S6K2 but not S6K1. Taking those criteria into account, we identified 14 unique protein signatures exclusive to S6K2. As shown in Table I, S6K2 sequence contains three signatures of the high mobility group proteins (HMGY and HMG14/17), two of which reside in S6K2 C-terminus while the third resides in S6K2 N-terminus. We also identified three signatures homologous to homeobox domain, two HOX9 activation region signatures and a prospero-like homeobox domain. Furthermore, we identified six protein signatures of regulation of pre-nuclear mRNA protein all of them resides in the extreme C-terminus. Interestingly those motifs are of proteins connected to chromatin and/or gene transcription regulation. The High-Mobility Group (HMG) proteins are architectural DNA and nucleosome-binding proteins that are involved in chromatin organisation and gene regulation [Pogna et al., 2010]. HOX9 activation region is a domain which constitutes the N-terminal of the paralogous homeobox proteins HoxA9, HoxB9, HoxC9 and HoxD9. This region is thought to act as a transcription activation region. Activation may be by interaction with proteins such as Btg proteins, which are thought to recruit a multi-protein Ccr4-like complex [Prevot et al., 2000]. The nuclear pre-mRNA regulation signatures can recognise the C-terminal domain (CTD) of the large subunit of RNA polymerase II. CTD is a platform for mRNA processing factors and links gene transcription to mRNA capping, splicing and polyadenylation. CTD recognition is dependent on the phosphorylation state of the CTD itself, which varies during the course of transcription but has also been linked to the isomerisation state of the CTD′s proline residues. Several RNA-processing factors recognise the CTD by means of a conserved CTD-interacting domain (CID). Factors with CID domains include the serine/arginine-rich-like factors SCAF4 and SCAF8, Nrd1 (which is implicated in polyadenylation-independent RNA 3′-end formation) and Pcf11. Pcf11 is a conserved and essential subunit of the yeast cleavage factor 1A, which is required for 3′-RNA processing and transcription termination [Meinhart and Cramer, 2004].

Taken those results together, the presence of those motifs and protein signatures simultaneously with the predominant nuclear localisation of S6K2 raised a possibility that S6K2 may have chromatin/transcription related functions. This could happen

TABLE I. Identification of New Motifs in S6K2 Sequence Using MOTIF Search Analysis Bioinformatic Tools

Motif ID	Motif description	Position (score)	Sequence	Function of source protein
IPB000116A IPB000116E	High mobility group protein (HMGY) signature	472.482 (1,106) 10.22 (1,096)	KSKRGRGRPGR ETEEGSEGEGEPEL	Non-histone chromosomal proteins. It is suggested that these proteins could function in nucleosome phasing and in the 3′-end processing of mRNA transcripts. They are also involved in the transcription regulation of genes containing, or in close proximity to A + T-rich regions
IPB000079	High mobility group protein HMG14 and HMG17	452.482 (1,059)	PPPPPSTTAPLPIRPPSGTKKSKRGRGRPGR	Non-histone chromosomal proteins. Bind to the inner side of the nucleosomal DNA thus altering the interaction between the DNA and the histone octamer. May be involved in the process which maintains transcribable genes in a unique chromatin conformation
PB006711D	Hox9 activation region	234.265 (1,129) 407.438 (1,086)	YMAPEILVRSGHNRAVDWWSLGLALMYDMLTG KLRSPRRLNSSPRAPVSPLKFSPFEGFRPSPS	Sequence-specific transcription factor. Contains a homeobox DNA-binding domain
IPB003417A	Core binding factor, beta subunit	342.368 (1,075)	DPPFRPCLGSEEDVQFDTRQTPV	CBF binds to the core site, 5′-PYGPYGGT-3′, of a number of enhancers and promoters, including murine leukaemia virus, polyomavirus enhancer, T-cell receptor enhancers, LCK, IL3 and GM-CSF promoters. CBFB enhances DNA binding by RUNX1
IPB007738B	Homeobox prospero-like	446.458 (1,062) 444.456 (1,060)	PLPPLIPPPPPST ELPIPPLLPPPPP	Contains a prospero-type homeobox DNA-binding domain. RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity
IPB006569B	Regulation of nuclear pre-mRNA protein	448.472 (1,098) 433.457 (1,096) 436.460 (1,090) 440.464 (1,063) 446.470 (1,060) 444.468 (1,057)	PPLLPPPPPSTTAPLPIRPPSGTKK FRPSPSLPEPTELPLPPLLPPPPPS SPSLPEPTELPLPPLLPPPPPSTTA PEPTELPLPPLLPPPPPSTTAPLPI PLPPLLPPPPPSTTAPLPIRPPSGT ELPLPPLLPPPPPSTTAPLPIRPPS	Interacts with phosphorylated C-terminal heptapeptide repeat domain (CTD) of the largest RNA polymerase II subunit POLR2A, and participates in dephosphorylation of the CTD. May act as a negative regulator of cyclin-D1 (CCND1) and cyclin-E (CCNE1) in the cell cycle

Amino acid sequence of S6K2 was inspected using MOTIF: a Sequence motif search in GenomeNet Bioinformatics Tools (http://www.genome.jp/tools/motif). Shown are examples of unique signatures that were identified in S6K2 but not S6K1.

through a direct interaction and localisation to chromatin and may also hint at possible involvement in transcription control through those substrates. Functional and mutational analysis of those motifs should reveal their physiological relevance in cells.

S6K2 IS BOUND TO CHROMATIN IN VIVO AND IS ATTACHED TO THE NUCLEAR MATRIX

S6 kinase 2 C-terminal regulatory region possesses a number of distinct features compared to its relative kinase; S6K1. S6K2 has a nuclear localisation and proline rich motif in addition to a number of regulatory phosphorylation sites. Here, we report an extra feature of S6K2 C-terminus region; it contains a number of unique signatures of nuclear proteins linked to chromatin such as HMG protein signatures. This prompted us to assess whether S6K2 binds to chromatin and/or the nuclear matrix. To achieve that we used a chromatin binding biochemical assay in HEK293 cells. The chromatin binding assay employs the extraction of cells with non-ionic detergents, such as Triton X-100, in order to enrich for chromatin bound proteins. Further treatment of Triton X-100 resistant chromatin binding proteins with DNase I and salt is needed to extract the chromatin bound proteins. The remaining insoluble fraction comprises the nuclear matrix proteins (see the Materials and Methods Section for details). A brief description of chromatin binding assay and isolated fractions is illustrated in Figure 1A. To achieve that, three sets of HEK293 cells were used: (1) TREX-HEK293 overexpressing EE-S6K2 cells were starved for 24 h, (2) exponentially growing TREX-HEK293 cells and (3) exponentially growing HEK293 cells. Cells were processed by chromatin binding assay and the generated fractions were analysed by Western blotting. EE-S6K2 levels were examined using anti-EE antibody, while endogenous S6K2 was analysed with anti-S6K2 polyclonal antibodies. The purity of the fractions was tested by anti-lamin A/C (the predominant protein in the nuclear matrix and a marker for nuclear proteins) and anti-β-tubulin (cytoplasmic marker) antibodies. As shown in Figure 1B, EE-S6K2 and endogenous S6K2 were detected in chromatin bound fractions (P1 and S2 fractions) as well as in the nuclear matrix fractions (P2). Notably, the phosphorylation of rpS6 at S240/244 was only detected in total and cytoplasmic fractions (T and S1 fractions).

Taken together, these data demonstrate the association of S6K2 with chromatin and nuclear matrix cellular fractions. This proximity of S6K2 to chromatin could be through either its chromatin binding motif or through its interaction with chromatin bound proteins as p300. The presence of S6K2 in the nuclear matrix fractions suggests its attachment to proteins that bind MARs/SARs regions of DNA and raise the possibility of the presence of unidentified S6K2 substrates at the chromatin level.

IN VITRO PHOSPHORYLATION OF HISTONES BY S6K2 WITH PREFERENTIAL ACTIVITY TOWARDS HISTONE H3 IN NUCLEOSOMAL STRUCTURE

Having established that S6K2 is bound to chromatin and nuclear matrix fractions, we raised the possibility of the presence of S6K2 substrates in those cellular compartments. For that, we ran a bioinformatic analysis of nuclear proteins related to transcriptional regulation which revealed a number of proteins, including histones, with the consensus sequence conforming to S6K phosphorylation

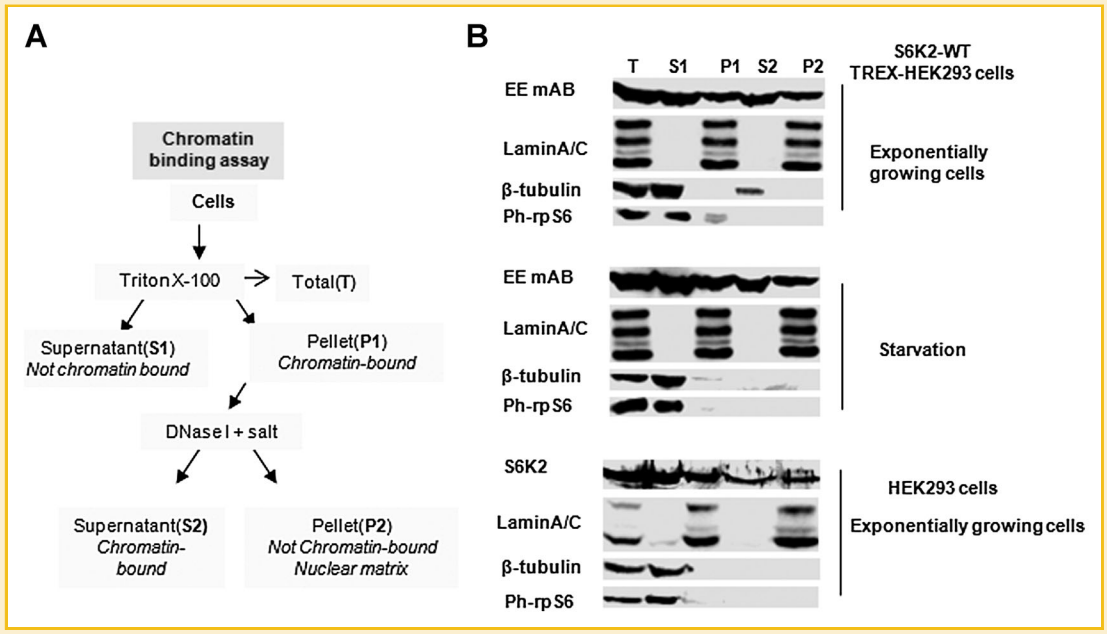

Fig. 1. S6K2 is bound to chromatin and nuclear matrix cellular fractions in vivo. A: A summary of chromatin binding assay and the identification of isolated cellular fractions (detailed in the Materials and Methods Section). B: TREX-S6K2 stable cells were induced with 1 μg/ml tetracycline for 6 h. Cells were then starved for 24 h. Exponentially growing HEK293 cells were also used. After harvesting, cells were subjected to chromatin binding assay and the isolated fractions were analysed by Western blotting to examine the fractionation patterns of S6K2 using anti EE antibody or anti-S6K2 polyclonal antibody. The activity of S6K was analysed by anti-phospho rpS6 (pS240/244) antibodies. Nuclear and cytoplasmic markers, lamin A/C and tubulin, respectively were used to determine the purity of the fractions. The results represented here were reproduced in three independent experiments.

motifs (RXRXXT/S) (Fig. 2A, Supplementary Table 1). In order to verify whether histones could be substrates for S6K2 activity, we applied the classical approach of in vitro kinase assays. Briefly, 1 μg of recombinant S6K2-His was incubated with one of the purified histones: H2A, H2B, H3 or H4 (1 μg each) for 30 min at room temperature. After the kinase assay, ^{32}P incorporation into histones was detected using a phosphoimager. The levels of recombinant histones were examined by Coomassie staining of the used gel. As shown in Figure 2B, histones H2A, H2B and H3 were phosphorylated by S6K2. However, although sufficient protein concentration of H4 was used in the reaction (as detected by Coomassie staining), no phosphorylation signal was observed (lane 4). S6K2 autophosphorylation signal was also observed and showed the highest level when S6K2 was incubated with histone H3 (lane 3). Quantification of the histone phosphorylation and S6K2 autophosphorylation is shown in the bar chart. These data suggest that S6K2 is able to phosphorylate core histones H2A, H2B and H3 in vitro, but is not able to phosphorylate histone H4.

We then questioned if these three histones would be phosphorylated whilst incorporated in the nucleosomal structure. Nucleosomes are formed from four dimers of core histones with two turns of DNA wrapped around them. In this structure, the accessibility of S6K2 to phosphorylation sites would play a great role, as the presence of the core histones in dimers or the presence of DNA could mask the phosphorylation sites. To test this, purified nucleosomes and histone H3 from calf thymus were used in S6 kinase assays as described above. Three phosphorylated bands in the nucleosomal mixture were expected, consistent with the phosphorylation of histones H2A, H2B and H3 as demonstrated above (Fig. 2B). However, only one phosphorylated band was obtained in lane 1, corresponding to histone H3 (Fig. 2B, right panel), while the histones were shown to be intact and in equal proportions as shown in the Coomassie stained gel below the autoradiograph.

Taken together, the above results demonstrate the ability of S6K2 to phosphorylate core histones in vitro. However, a selective phosphorylation of histone H3 is observed in nucleosomes. This could be attributed to the accessibility of core histones within the nucleosomal structure. The site, which is phosphorylated in histone H3, could be exposed, while the other sites in histones H2A and H2B were masked by their presence in dimers or in nucleosome structures. Furthermore, the presence of DNA turns could mask those phosphorylation sites. This preferential phosphorylation of histone H3 in nucleosome structure by S6K2 prompted us to focus on the identification of this phosphorylation site and elucidate the conditions for phosphorylation in vivo.

S6K2 BUT NOT S6K1 OR AKT IS ESSENTIAL FOR HISTONE H3-THR45 ACTIVATION

After showing that histone H3 can be phosphorylated in vitro by S6K2, we next assessed if S6K1 can also phosphorylate histone H3. The efficiency of both enzymes to phosphorylate rpS6 and histone H3 was examined using an in vitro kinase assay. To achieve this, wild-type and constitutively active mutants (T412D and T401D) of S6K1 and S6K2 were incubated with either 80S rpS6 or histone H3 for 30 min at room temperature. After the kinase assay, ^{32}P incorporation into rpS6 or histone H3 was detected using a phosphoimager.

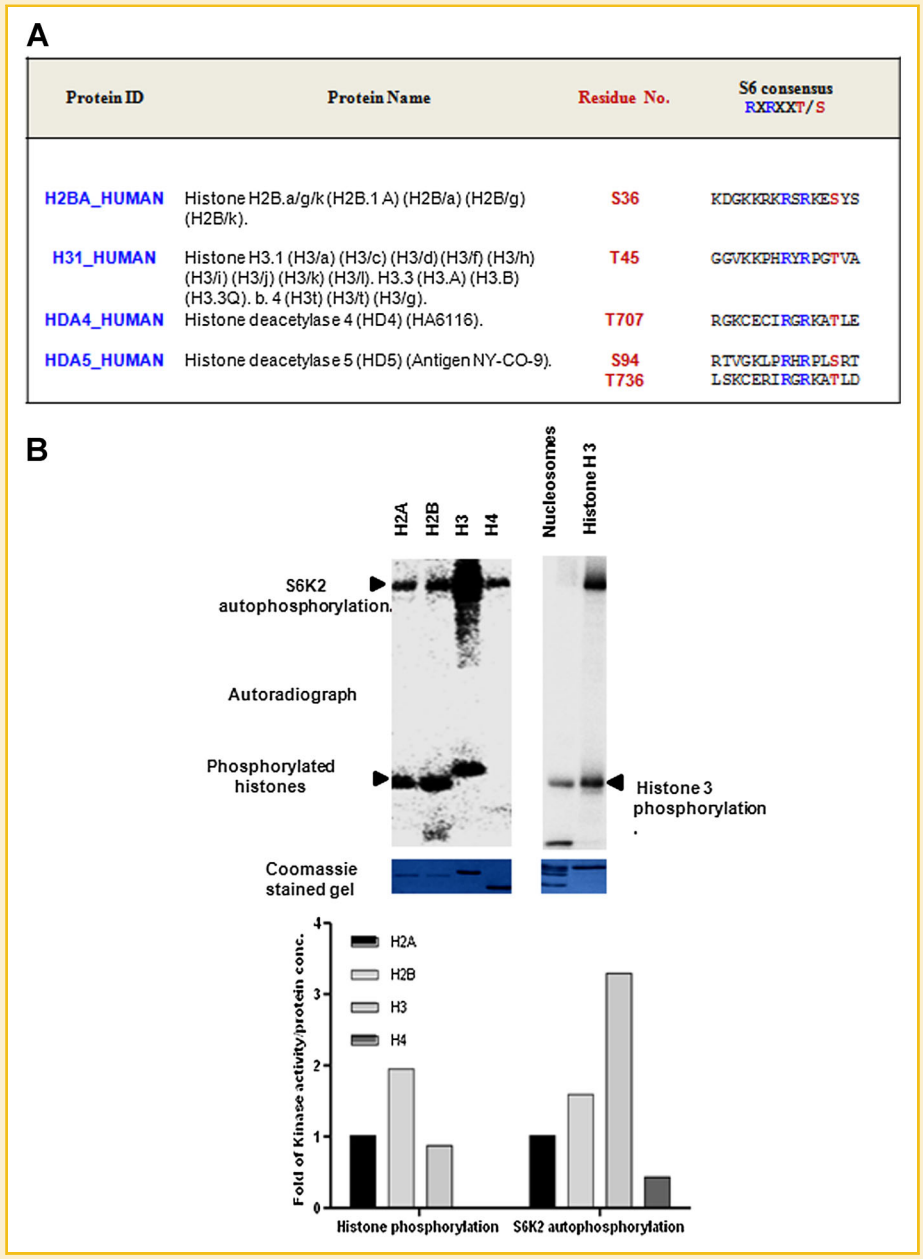

Fig. 2. S6K2 phosphorylates recombinant histones in vitro. A: Sequence analysis of histone H2B and H3 reveals the presence of S6K consensus sequence. B: S6K in vitro kinase assay. Recombinant His-tagged S6K2 was incubated in a 32P in vitro kinase assay with recombinant core histones (histones H2A, H2B, H3 and H4) (left panel) or purified nucleosomes and calf thymus histone H3 (right panel). Samples were resolved by SDS–PAGE and ^{32}P incorporation into histones was determined using a phosphoimager. Coommassie staining of the analysed gels confirmed the integrity of the histones used. The graph shows folds of the kinase activity measured by densitometric analysis of histones phosphorylation and S6K2 autophosphorylation. S6K2 activity towards histone H2A was set as unity. The results represented here were reproduced in three independent experiments.

Generation of constitutively active mutants of S6K1 (T412D) and S6K2 (T401D) have been previously described [Valovka et al., 2003]. These mutants were generated by substituting Thr412 in S6K1 and Thr401 in S6K2 for aspartic acid. These mutations mimic the presence of a phosphate group and increase S6K1/2 kinase activities when compared to wild-type kinases. As shown in Figure 3A, S6K2, wild-type and activated mutant (T401D) is more efficient in phosphorylating histone H3 compared to phosphorylation of rpS6 (Fig. 3A). Wild-type and activated mutants (T412D) of S6K1, can phosphorylate histone H3 in vitro, but work less efficiently when compared with the classical substrate rpS6, suggesting a preference for rpS6 as a substrate (Fig. 3A). The bar chart shows the kinase activity fold of each protein on ribosomal proteins and histone H3.

Inspection of the histone H3 protein sequence revealed the presence of a threonine residue (Thr45), which could be phosphorylated by S6K2, as it lies in the consensus sequence RXRXXT which

conform to the S6K substrate ribosomal S6 proteins (see Fig. 2A). It is important to note that Thr45 is located within the histone fold domain (Fig. S1). The phosphorylation of this site was described recently by three groups [Hurd et al., 2009; Baker et al., 2010; Villagrasa et al., 2012]. Having established that recombinant S6K2 has a much higher affinity towards histone H3 than its relative kinase homologue S6K1 in vitro, we then investigated if the phosphorylation site in H3 recognised by S6K2 in vitro is Thr45. The availability of commercial phosphospecific antibodies against histone H3-Thr45 (anti H3-pThr45 antibody) allowed us to test the phosphorylation of this residue in vivo.

We detected histone H3-Thr45 phosphorylation in exponentially growing HEK293 cells, which prompted us to question whether this site is specifically phosphorylated in vivo by S6K2. AKT is also known to phosphorylate Thr/Ser in the same motif (RXRXXT/S) and therefore might also be involved in mediating H3-Thr45 phosphorylation in vivo. To address this issue, RNAi mediated knockdown of the three kinases (S6K1, S6K2 and AKT) was applied. siRNA duplexes targeting S6K1, S6K2 and AKT in addition to a scrambled siRNA control were transfected separately into HEK293 cells (see the Materials and Methods Section for details). Knockdown was allowed to take place for 72 h, and cells were then harvested in 1× sample buffer. The efficiency of protein knockdown was examined using specific antibodies towards S6K1, S6K2 and AKT. H3-Thr45 phosphorylation was monitored by Western blotting using anti H3-pThr45 antibody. Equal loading of proteins was assessed by anti-actin antibody. As shown in Figure 3C, all three kinases were shown to be successfully knocked down compared with the scrambled RNAi control. Thr45 phosphorylation was significantly reduced when knocking down S6K2. Conversely, when S6K1 or AKT were knocked down, the phosphorylation at this site was not significantly affected. These results reveal that S6K2, but not AKT or S6K1, is involved in histone H3-Thr45 phosphorylation in HEK293 cells.

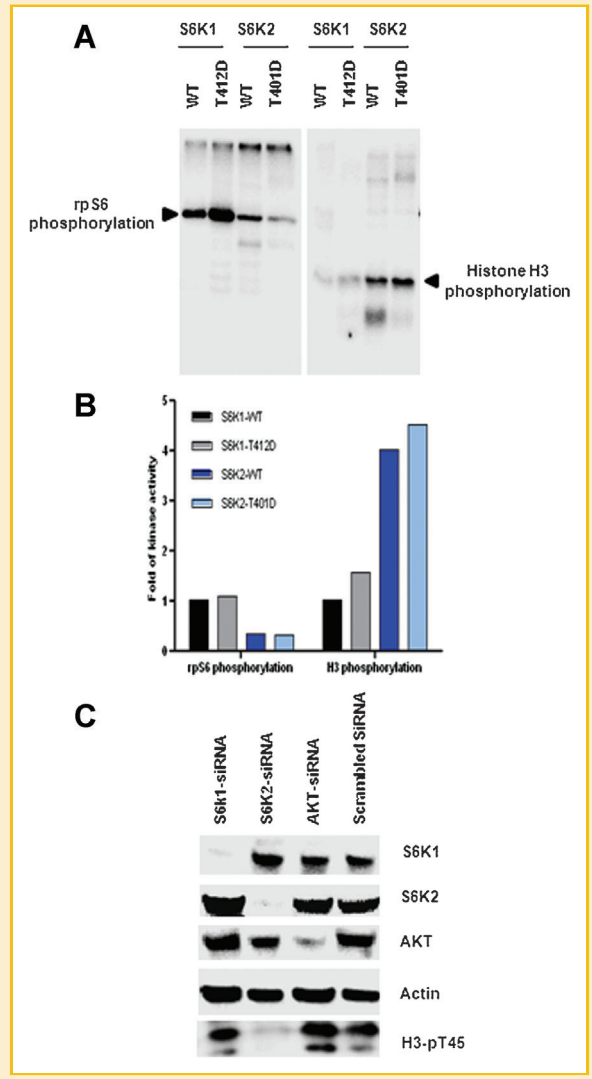

Fig. 3. S6K2 but not S6K1 or AKT is essential for H3-Thr45 activation in HEK293 cells. A: In vitro kinase assay: recombinant His-tagged proteins S6K1WT, S6K2WT, S6K1-T412D or S6K2-401D were incubated in a ^{32}P in vitro kinase assay with 80S ribosomes as the source of rpS6 substrate or with calf thymus histone H3. Samples were then resolved by SDS–PAGE and ^{32}P incorporation into rpS6 or H3 proteins was determined using a phosphoimager. B: Graph shows folds of kinase activity were measured by densitometric analysis of rpS6 and H3 phosphorylation. S6K1 activity towards rpS6 or H3 was set as unity. C: Knockdown of S6K2 abolishes phosphorylation of H3-T45. HEK293 cells were transfected with RNA duplexes towards S6K1, S6K2 or AKT using lipofectamine 2000 for 72 h. In one set scrambled RNA duplexes were used as control. Cells were collected in PBS containing protease inhibitors and sonicated in 1× protein loading buffer. Samples were then examined by Western blotting for knockdown of S6K1, S6K2 or AKT using specific antibodies. Thr45 phosphorylation was assessed using anti-H3-pT45 phosphospecific antibody. Equal loading of proteins was tested using anti-actin antibody. These findings were reproduced in more than five independent experiments.

NUCLEAR LOCALISATION OF S6K2 IS ESSENTIAL FOR H3-THR45 PHOSPHORYLATION

S6K2 isoforms have been shown to predominantly localise to the nucleus, as they contain a nuclear localisation sequence (NLS) in the C-terminus compared with S6K1 isoforms. Stable TREX-HEK293 cells overexpressing S6K2-WT or NLS-mutant were subjected to subcellular fractionation. The distribution of EE-tagged proteins in cytoplasmic and nuclear fractions was analysed by Western blotting using anti-EE mAb. The purity of each fraction was analysed with specific nuclear and cytoplasmic protein markers. Lamin A/C, a nuclear structural protein was used as a marker for the presence of nuclear proteins, and β-tubulin (microtubular protein) as a marker for the cytoplasm. As shown in Figure 4A, NLS-mutant showed a considerable shift of the protein to the cytoplasm rather than the nucleus when compared with the S6K2 wild type. Notably, 60% of S6K2 wild-type protein was detected in the nuclear fractions while only 18% of NLS-mutant protein was detected in nuclear fractions in exponentially growing cells.

Having established that S6K2 phosphorylates histone H3 at Thr45, we next assessed if the S6K2 NLS would play a role in this phosphorylation. To achieve that, TREX-HEK293 cells overexpressing S6K2WT or NLS-mutant were lysed and exogenous proteins were immunoprecipitated using anti-EE antibody. Immune complexes were then used in S6K in vitro kinase assays in the presence of recombinant H3 or 80S ribosomal proteins as a substrate. The efficiency of immunoprecipitation samples was tested using anti EE

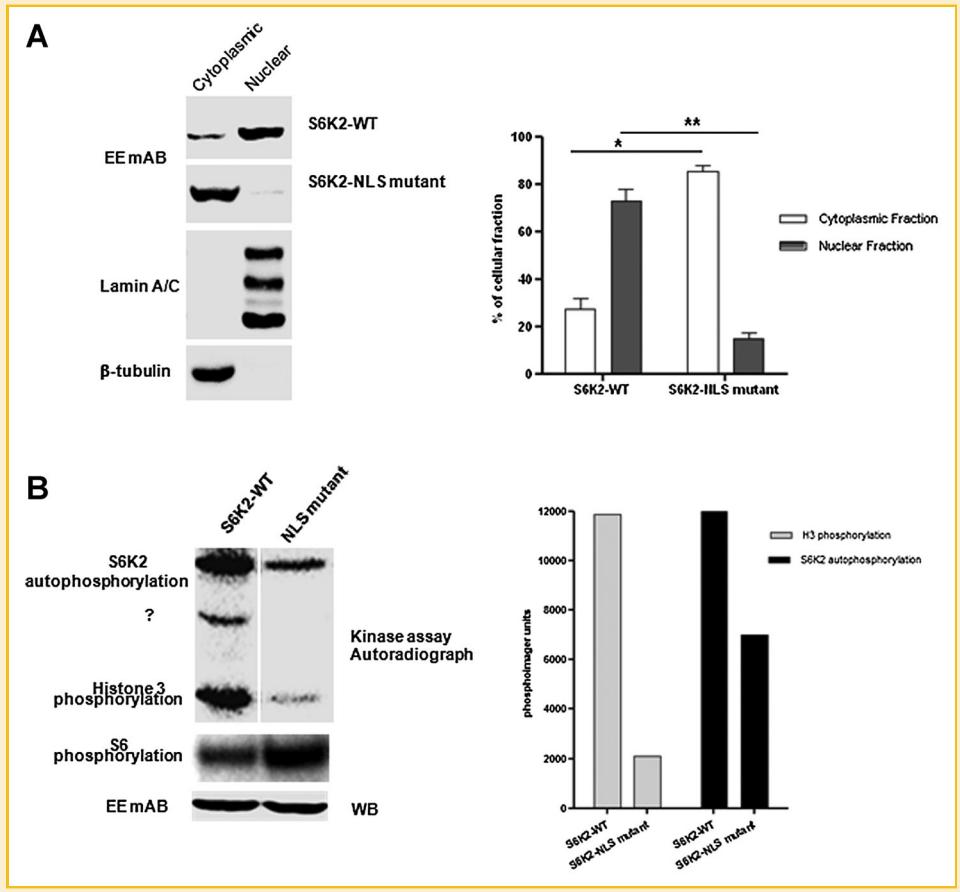

Fig. 4. Disruption of the S6K2 nuclear localisation sequence affects phosphorylation of histone H3 at Thr45. A: Nuclear fractionation: TREX-S6K2WT or TREX-NLS mutant stable cells were induced with 1 μg/ml tetracycline for 12 h. Cells were harvested in hypotonic buffer for 30 min. The supernatant was then centrifuged for 2 min at 800*g*. The supernatant was then removed and retained as the cytoplasmic fraction. The pellet was resuspended in EB buffer and incubated for 30 min on ice. The sample was then centrifuged at 10,000*g* for 30 min. The remaining supernatant is the nuclear fraction. Nuclear and cytoplasmic markers, lamin A/C and tubulin, respectively were used to determine the purity of the fractions. The graph shows the densitometric analysis for fractions ± SEM. The results were analysed with two-tailed paired *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.001$. B: In vitro kinase assay. TREX-S6K2WT and TREX-NLS mutant stable cells were induced with 1 μg/ml tetracycline for 12 h. After harvesting, cells were lysed and EE-S6K2 was immunoprecipitated from total cell lysates using anti-EE antibody. Immunocomplexes were extensively washed and used in S6K ^{32}P in vitro kinase assays using calf thymus H3 or ribosomal proteins as a substrate. Samples were resolved by SDS–PAGE and ^{32}P incorporation into H3 was determined using a phosphoimager. The graph chart represents the Thr45 phosphorylation and S6K2 autophosphorylation signals that were measured in phosphoimager units. The results represented here were reproduced in three independent experiments.

antibody. As shown in Figure 4B, Histone 3 was phosphorylated by S6K2 wild type and NLS-mutant with different efficiencies. S6K2 wild type was able to phosphorylate H3 with high efficiency, while this phosphorylation was significantly reduced by more than 80% when using NLS-mutant as the phosphorylating kinase. Notably, the addition of H3 to the kinase assay strongly induced S6K2 autophosphorylation. S6K2 WT and NLS mutant were able to phosphorylate S6 proteins and the NLS-mutant showed higher efficiency. These data indicate that disruption of the S6K2 NLS affected phosphorylation of the Thr45 residue in histone H3. This effect of S6K2 mutant is not related to misfolded protein as S6K2-NLS mutant was efficient in phosphorylating rpS6.

The results shown here highlight the importance of the NLS of S6K2 in the efficiency of H3-Thr45 phosphorylation, which could be attributable to the requirement of the full NLS-sequence to ensure proper interaction between S6K2 and histone H3 and achieve maximum activation.

HISTONE 3 THR45 PHOSPHORYLATION CORRELATES WITH S6K ACTIVATION IN RESPONSE TO MITOGENS

Having established that the histone H3-Thr45 residue is phosphorylated in vitro and in vivo by S6K2, we then investigated the physiological conditions regulating this phosphorylation event. Histone phosphorylation was initially linked to chromosome condensation; H3 post-translational phosphorylation can be divided into two categories: inducible and mitotic. Inducible phosphorylation could be due to mitogens and stresses. We first studied the effect of serum withdrawal and addition on Thr45 phosphorylation in histone H3 in NIH3T3 cells. Briefly, NIH3T3 cells were starved for 24 h, and then stimulated with FBS for 1 h or stimulated in the presence of 50 nM rapamycin for the indicated times. Cells were then harvested in 1× sample buffer and analysed by Western blotting for Thr45 phosphorylation using anti H3-pT45 antibody. The phosphorylation/activation status of S6K was tested using anti-pT412-S6K antibodies. As shown in Figure 5A, the phosphorylation of H3-Thr45 is induced

by serum and is almost completely halted by serum starvation. The pattern of phosphorylation of Thr45 phosphorylation correlates with that of S6K activation/phosphorylation at T412 when rapamycin is introduced in a longer time course experiment (left panel) (*anti-pT412-S6K blot*). Equal loading of proteins were assessed by immunoblotting with anti-actin antibody.

HISTONE H3-THR45 IS PHOSPHORYLATED DURING TPA-INDUCED DIFFERENTIATION OF LEUKAEMIC CELL LINES

High expression levels of S6K2 in promyelocytic HL-60, monocytic U937 and monocytic like THP-1 cells (data not shown), have prompted us to study H3-Thr45 phosphorylation profile in these cell lines. HL-60, U937 and THP-1 are characterised by their ability to differentiate into macrophages in response to 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) [Breitman et al., 1980; Bar-Shavit et al., 1983; Welgus et al., 1986]. S6 kinase is known to be activated in the above cell lines during differentiation [Gomez-Cambronero et al., 2003]. We wanted to ascertain the role of histone H3-Thr45 phosphorylation during differentiation in relation to S6K activation. To this end, the differentiation of U937, HL-60 and THP-1 cells was induced by 10 nM TPA. All the above cell lines adhere to culture plates when differentiation begins. U937 and THP-1 cells differentiate in 72 h and their morphology changes from round to spindle shaped; HL-60 cells differentiate after 48 h of treatment and begin to clump together (data not shown). After the induction of differentiation, cells were harvested and analysed by Western blotting for H3-Thr45 phosphorylation using anti H3-pThr45 antibody. Equal protein loading was checked using anti-actin antibody. Protein levels of S6K2 were assessed using anti-S6K2 antibody. The activation/phosphorylation status of endogenous S6K was measured by the phosphorylation at T412 using anti-pT412 S6K antibodies, while the in vivo activity of S6K was measured by rpS6 phosphorylation using anti-phospho rpS6 antibodies. As shown in Figure 6A, histone H3-Thr45 phosphorylation in U937 cells is induced after 48 and 72 h of differentiation, in parallel with S6K activation measured by phosphorylation at T412. The phosphorylation of rpS6 was induced at S240/244, which is mediated by S6 kinases. The same pattern of induction of H3-Thr45 was observed in the HL-60 cells, as phosphorylation at that site was induced after 48 h, in parallel to S6 kinase activation and S6 protein phosphorylation status (Fig. 6B). As reported by Kramer et al. [2002], monocyte/macrophage differentiation of HL-60 leukaemia cells requires induction of the cyclin-dependent kinase inhibitor p21 (WAF1/CIP1), and cell cycle arrest at the G1/S checkpoint. For that, the induction of p21 protein expression level was used as a marker for differentiation in this experiment. The results demonstrated here confirm that histone H3-Thr45 phosphorylation is stimulated after TPA-induced differentiation of U937 and HL-60 cells.

We additionally tested the status of H3-Thr45 phosphorylation in monocyte-like THP-1 cell line. As shown in Figure 6C, S6K and mTOR phosphorylation is induced in TPA-differentiated cells compared to undifferentiated controls. H3-Thr45 phosphorylation was higher in differentiated cells than undifferentiated but there was a basal level of phosphorylation in the control set. The differentiation of THP-1 was confirmed by the expression of CD36 gene in differentiated cells (Fig. 6C, bar chart). As the level of H3-Thr45 was higher in undifferentiated THP-1 cells compared with undifferentiated HL60 and U937, we set up a quantitative assay to detect this phosphorylation by flow cytometry. THP-1 cells were differentiated with TPA for 72 h and the phosphorylation of H3-Thr45 was analysed by FACS. As shown in Figure 6D, a high basal level of H3-Thr45 phosphorylation was detected in undifferentiated cells compared to unstained control cells. A significant increase in the mean fluorescence intensity of H3-Thr45 phosphorylation was detected in response to TPA differentiation (Fig. 6D, histograms and bar chart). To test the effect of rapamycin on the H3 phosphorylation in this cell line, the above experiment was repeated in the absence and presence of 50 nM rapamycin. As shown in Figure 7A, rapamycin was effective in reducing mTOR phosphorylation in response to TPA (blots), and blocked the observed induction of H3-Th45 phosphorylation. The same experiment was repeated and cells were analysed by flow cytometry. Interestingly, we obtained the same results of Western blotting as shown in Figure 7A (histogram and bar chart). Rapamycin significantly reduced the H3-Thr45 phosphorylation to the basal levels observed in undifferentiated cells, however no effect was observed in undifferentiated cells. To test the effect of rapamycin on the differentiation of THP-1 cells, we

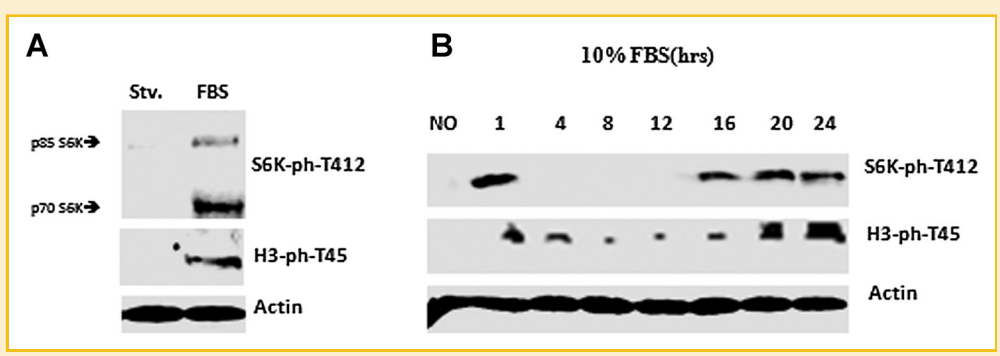

Fig. 5. Histone H3-Thr45 phosphorylation correlates with S6K activation in response to mitogens. A: In vivo phosphorylation of histone H3 at Thr45 in NIH3T3 cells. NIH3T3 cells were starved for 24 h and then stimulated with 10% FBS for 1 h. B: For rapamycin treatments, cells were starved for 24 h then stimulated with FBS for the indicated times in the presence of rapamycin. After harvesting, cells were lysed in 1× sample buffer and analysed by Western blotting. Thr45 phosphorylation was assessed using anti-H3-pT45 phosphospecific antibody. The S6K activation/phosphorylation status was analysed using anti-S6K-pT412 antibodies.

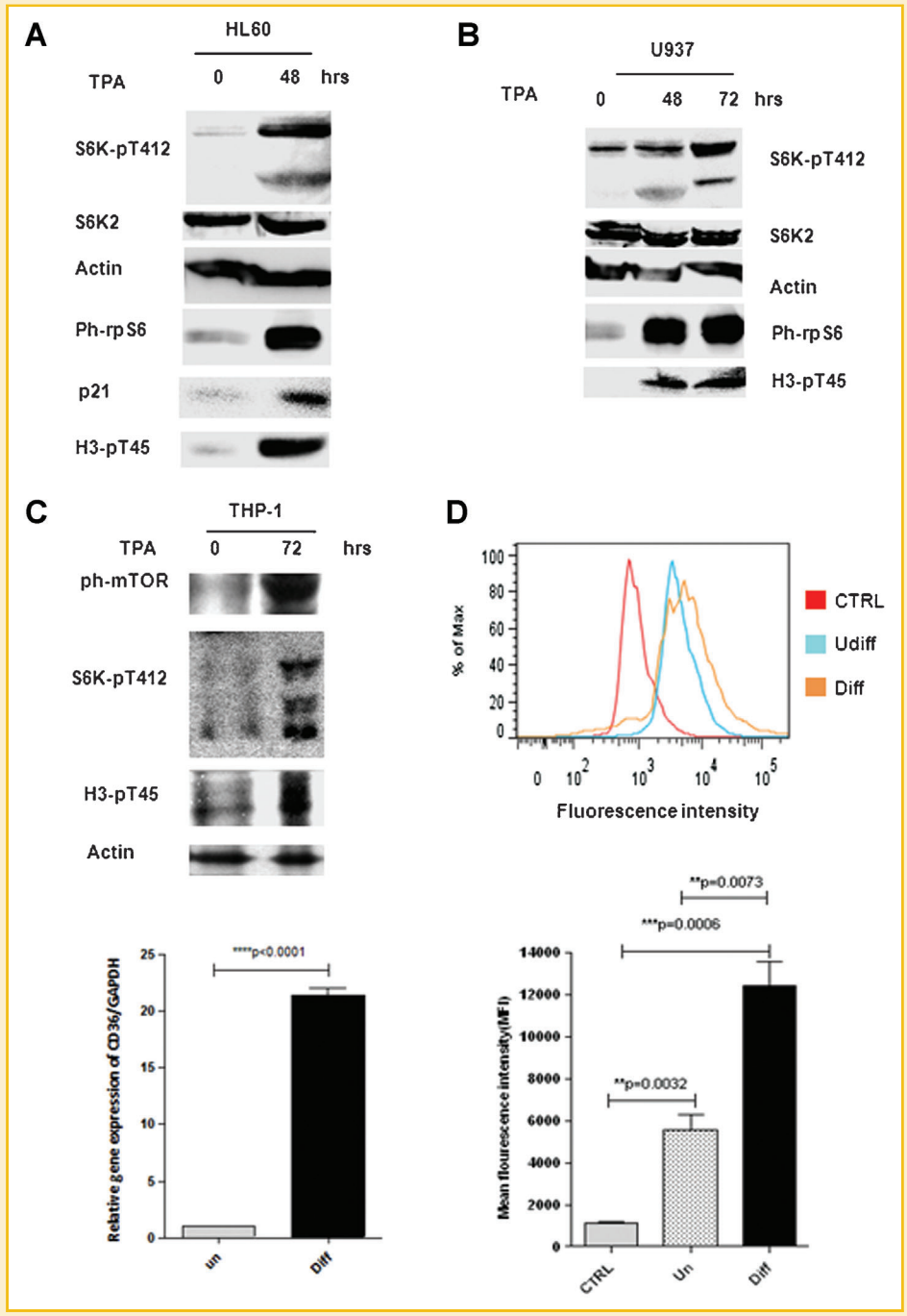

Fig. 6. H3-Thr45 phosphorylation correlates to S6K activation in response to TPA-induced cell differentiation of leukaemic cell lines. In (A–C) exponentially growing HL60, THP-1 and U937 were set for differentiation by induction with TPA (10 nM). Cells were incubated with TPA for the indicated times. After differentiation, the adhered cells were washed in 1XPBS, lysed in 1× sample buffer and analysed by Western blotting. Activation of S6K was measured by phosphorylation at T412 and S6 protein phosphorylation. H3-Thr45 phosphorylation was detected by specific antibodies. Differentiation of HL60 cells was confirmed with the detection of p21 expression. C: Graph shows the gene expression levels of CD36 gene in undifferentiated and differentiated cells as a marker for Differentiation of THP-1 cells. Gene expression levels were normalised against GAPDH. D: Flow cytometry. THP-1 cells were treated with 10 nM TPA or DMSO for 72 h. Phosphorylation of H3-Thr45 was detected as described in methods. CTRL: unstained control cells, Undiff: undifferentiated, Diff: TPA differentiated cells. Histogram represents the fluorescence values of each set. Mean fluorescence intensity (MFI) values for all set were graphed and analysed by *t*-test. Triplicates of each time point was used in flow cytometry analysis.

microscopically monitored the morphology of the cells after treatment with TPA in the absence and presence of rapamycin. We additionally investigated the effect of rapamycin on TPA-induced expression of CD36 gene by qRT-PCR. As shown in Figure 7B, no obvious differences were observed in the cells morphology after TPA-induced differentiation in the presence or absence of rapamycin. Rapamycin treatment caused a reduction of CD36 gene expression compared to TPA only treated cells, however this reduction did not show any statistical significance (Fig. 7C). These data indicate that rapamycin does not interfere with the differentiation process of THP-1 cells in response to TPA.

Taken those results together, we demonstrated a strong evidence of histone H3-Thr45 phosphorylation in leukaemic monocyte cell lines (U937, THP-1 and HL60) in response to TPA-induced differentiation to macrophages. This phosphorylation event is correlated with S6K pathway activation and implicates an important role of H3 histone fold phosphorylation transduced by S6K signalling in monocyte/macrophage differentiation.

DISCUSSION

Accumulating evidence suggests that the mTOR/S6K pathway is a crucial regulator of cell growth and metabolism. Deregulation of the mTOR/S6K signalling pathway may invoke a predisposition to the progression of cancer. The bulk of research published in this respect has focused on S6K1 as a key player downstream of mTOR, far less attention has been given to the closely related homologue S6K2. It was only recently that Pardo et al. [2006] identified a protein complex including S6K2, PKCepsilon and B-RAF that mediates cell survival in SCLC cells. This report could be considered the first to address the specific regulatory mechanisms that involve S6K2. Alternative splicing in S6K2 gene results in two isoforms which are both predominantly nuclear. This knowledge motivated us to investigate in more detail the essential role S6K2 plays in the nucleus and its physiological relevance.

S6K1 and S6K2 are two highly similar kinases which mostly differ in their C- and N-termini. This may indicate different modes of their

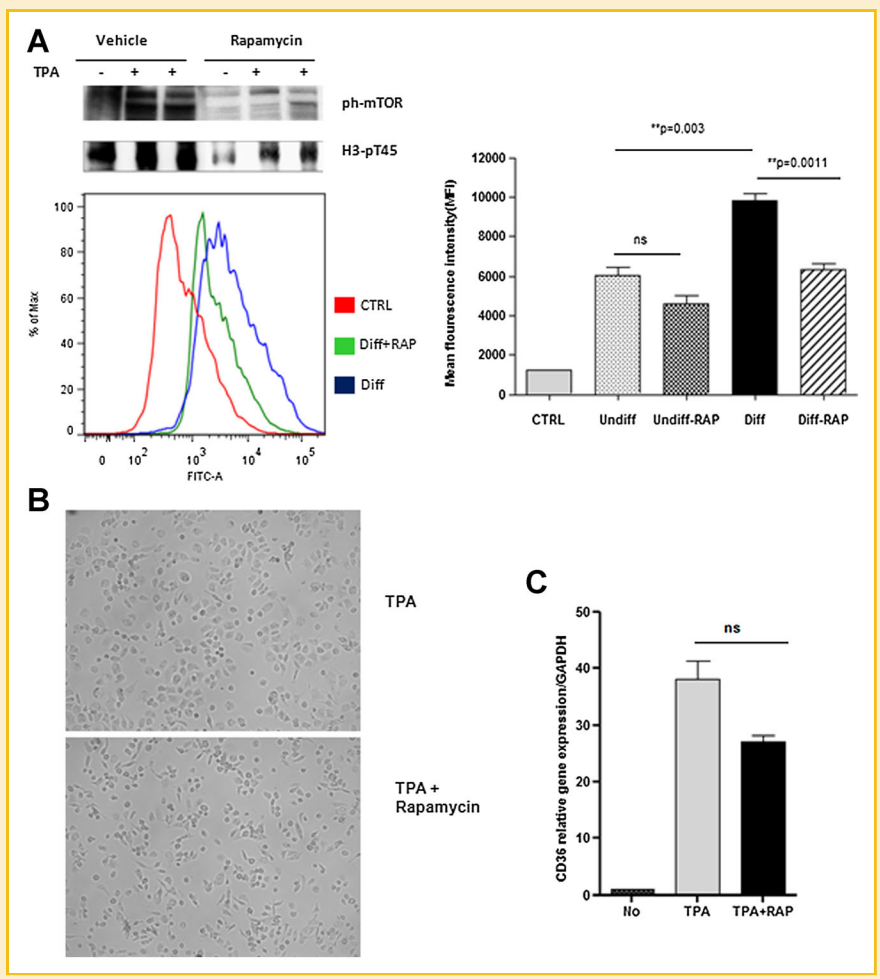

Fig. 7. Rapamycin inhibits TPA-induced histone H3-Thr45 phosphorylation in differentiating THP-1 cells. A: THP-1 cells were treated with TPA or DMSO in the presence or absence of 50 nM rapamycin. Differentiation allowed for 72 h. One set of cells was used for Western blotting analysis of H3-Thr45 phosphorylation and mTOR phosphorylation (blots) and another set was used for flow cytometry analysis using anti-phosphor H3-Thr45 (histograms and bar charts). B: Microscopic pictures of differentiating THP-1 cells in the presence or absence of 50 nM rapamycin. C: Graph shows the effect of rapamycin on gene expression levels of CD36 gene in undifferentiated and differentiated cells. Gene expression levels were normalised against GAPDH.

regulation. Common and distinct substrates were expected for S6 kinases when S6K2 was discovered. The presence of chromatin bound proteins related motifs exclusive to S6K2 at its C-terminus provides additional evidence that the two kinases behave differently in the cell (Table I). The motifs we identified are found in nuclear proteins with important functions in chromatin organisation and gene transcription, such as high mobility group protein family and homeobox domain containing proteins. This led us to investigate the possibility of S6K2 binding to chromatin. Interestingly, S6K2 was found in chromatin and nuclear matrix cellular fractions (Fig. 1). This finding provides a valuable clue as to the potential function of S6K2, suggesting that it may phosphorylate a chromatin-associated molecule and thus regulate a DNA-related process such as transcription, replication, recombination or the repair of DNA damage. This would greatly facilitate the search for S6K2-specific substrates at the chromatin level and would provide some clues regarding its function. The functional and mutational analysis of those motifs would provide new insights into the chromatin related functions of S6K2.

The establishment of a protein as an S6K substrate should be based on several criteria previously defined by Cohen and Frame [2001]: first, conformity of the sequence context of the phosphorylated serine or threonine with the S6K recognition motif (RXRXXT/S); second, in vitro phosphorylation by S6K; third, correlation of the in vivo phosphorylation with S6K activity; and last, but most stringently, involves the ability of S6K knockout or knockdown to confer hypophosphorylation on the putative substrate. We used a bioinformatic approach to search for nuclear proteins involved in transcription and chromatin function that carry the S6K consensus sequence (Supplementary Table 1). The attractive examples in that list are histones H3, H2B and TAFs.

The presence of an S6K phosphorylation consensus sequence in the histone fold domain of histone H3 (threonine 45) was of specific interest to us regarding the possible phosphorylation of this site in vivo. The phosphorylation of histones was verified by in vitro phosphorylation of individual histones using S6K in vitro kinase assays (Fig. 2). The selective phosphorylation of histone H3 in nucleosomes can be explained by the accessibility of the phosphorylation site in histone H3 which allows recognition by S6K2. This may resemble the situation in vivo, as histones are present as octamers and wrapped by DNA, which could hide some sites and expose others for modification. Another interesting observation is the induction of autophosphorylation of S6K2 in the presence of H3. It would be useful to identify the site of this autophosphorylation which could explain a mechanism of this event.

The finding that S6K2 is bound to chromatin in vivo and in vitro strengthens the hypothesis that S6K2 could be involved in the dynamic regulation of transcription. The chromatin bound state of S6K2 also suggests its proximity to nuclear proteins involved in transcription. This proximity could lead to the phosphorylation of nuclear substrates or restrict the access of other proteins for targeting transcription complexes. The proximity of S6K2 to chromatin would aid the interaction with histones, particularly histone H3 and its phosphorylation. The presence of S6K2 in nuclear matrix fractions could help in its binding complex protein bound to MARs and SARs DNA elements which help when anchoring proteins to DNA. The preference of S6K2, but not S6K1 for histone H3 as a substrate compared with the classical substrate rpS6 in vitro strengthened the finding that S6K2 might be responsible for phosphorylating histone H3 at Thr45 in vivo in HEK293 cells. Using phosphospecific antibodies towards H3-Thr45, we were able to confirm the phosphorylation at this site by S6K2 in vitro and in vivo. Disruption of the S6K2 nuclear localisation greatly affected the efficiency of S6K2 to phosphorylate histone H3 at Thr45, suggesting the involvement of NLS sequence in this signalling event. Interestingly, it was reported previously that nuclear staining of S6K2 increases in breast adenocarcinomas in comparison to normal tissue [Filonenko et al., 2004].

Knockdown of S6K1, S6K2 and AKT showed that S6K2 is the responsible kinase for Thr45 phosphorylation as its knockdown abolished phosphorylation of this site in HEK293 cells. The finding that Thr45 phosphorylation is induced upon serum stimulation and its reduction by rapamycin in NIH3T3 cells, links histone H3 function to signal transduction and the mTOR/S6K pathway in particular. In previous studies, histone H3 phosphorylation was reportedly induced by mitogenic stimulation, especially Ser10 and Ser28. Significant findings such as the observation that histone H3 phosphorylation (Ser10) is critical for neoplastic cell transformation [Dong and Bode, 2006] suggests that histone H3 may be a crucial target for cancer chemotherapy or genetic therapy in the future. Therefore, defining the pattern of Thr45 phosphorylation under physiological and pathological conditions might be useful for developing novel diagnostic and therapeutic approaches.

In a trial to identify the physiological context of Thr45 phosphorylation by S6K2, we found that induction of this site phosphorylation was detected in differentiated leukaemic cell lines U937, THP-1 and HL60 cells that were induced by TPA. We confirmed those results by Western blotting and flow cytometry in THP-1 cells. An interesting observation is the high basal level of H3-Thr45 phosphorylation in undifferentiated THP-1 cells compared to U937 and HL60 under the same culture conditions. This may explain why THP-1 is more aggressive leukaemic cell line compared to HL60 that is less malignant. Whether this could link H3-Thr45 phosphorylation to malignancy needs further investigation.

The ability of S6K2 to phosphorylate histone H3, in the histone fold domain, in addition to the presence of an S6K target sequence in histone fold containing proteins like TAFs (Supplementary Table 1), raises the possibility that S6K2 could phosphorylate histone fold containing proteins and that TAFs are potential substrates for this phosphorylation. The TAF subunits of TFIID mediate transcriptional activation of subsets of eukaryotic genes [reviewed in Selleck et al., 2001].

In summary, we describe the identification of S6K2 in chromatin bound fractions of HEK293 cells. Using in vitro kinase assays and RNAi mediated knockdown of S6K1, S6K2 and AKT, we showed that S6K2 is responsible for in vivo phosphorylation of Thr45. The physiological relevance of this modification by S6K2 is not clear at the moment, but Thr45 phosphorylation is readily induced by serum stimulation and TPA-driven differentiation of U937, THP-1 and HL60 monocyte leukaemic cell lines. The other aspects of H3-Thr45 phosphorylation via S6K2 under physiological and pathological conditions are currently under investigation.

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