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Histone acetyltransferases interact with and acetylate p70 ribosomal S6 kinases *in vitro* and *in vivo*

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ABSTRACT

The 70 kDa ribosomal protein S6 kinases (S6K1 and S6K2) play important roles in the regulation of protein synthesis, cell growth and survival. S6Ks are activated in response to mitogen stimulation and nutrient sufficiency by the phosphorylation of conserved serine and threonine residues. Here we show for the first time, that in addition to phosphorylation, S6Ks are also targeted by lysine acetylation. Following mitogen stimulation, S6Ks interact with the p300 and p300/CBP-associated factor (PCAF) acetyltransferases. S6Ks can be acetylated by p300 and PCAF *in vitro* and S6K acetylation is detected in cells expressing p300. Furthermore, it appears that the acetylation sites targeted by p300 lie within the divergent C-terminal regulatory domains of both S6K1 and S6K2. Acetylation of S6K1 and 2 is increased upon the inhibition of class I/II histone deacetylases (HDACs) by trichostatin-A, while the enhancement of S6K1 acetylation by nicotinamide suggests the additional involvement of sirtuin deacetylases in S6K deacetylation. Both expression of p300 and HDAC inhibition cause increases in S6K protein levels, and we have shown that S6K2 is stabilized in cells treated with HDAC inhibitors. The finding that S6Ks are targeted by histone acetyltransferases uncovers a novel mode of crosstalk between mitogenic signalling pathways and the transcriptional machinery and reveals additional complexity in the regulation of S6K function.

1. Introduction

Activation of the 70 kDa ribosomal S6 kinase (S6K1) in response to a variety of growth factors, cytokines, nutrients and cellular stresses results in the phosphorylation of multiple targets involved in protein synthesis and RNA metabolism, thus increasing the translational capacity of the cell and facilitating cell growth (Ruvinsky and Meyuhas, 2006). Mice and humans possess two *S6K* genes, *S6K1* and *S6K2*, which encode proteins with highly homologous kinase domains but divergent N- and C-terminal regulatory regions that appear to specify different protein–protein interactions and subcellular localization patterns to these paralogues (Gout et al., 1998; Saitoh et al., 1998; Shima et al., 1998; Koh et al., 1999; Lee-Fruman et al., 1999). A number of studies in which either or both *S6K* genes have been deleted in mice suggest that the control of cell growth is a non-redundant function specific to S6K1, while phosphorylation of the canonical target, ribosomal protein S6 (rpS6) can be mediated by both S6K1 and S6K2 (Shima et al., 1998; Pende et al., 2004). S6K1 may promote cell growth via the phosphorylation of several distinct substrates, including the eEF2 kinase, a regulator of translation and SKAR, a positive regulator of cell growth with presumed roles in RNA processing (Wang et al., 2001; Richardson et al., 2004). S6K1 may also enhance cell growth by directing the degradation of programmed cell death protein 4 (PDCD4), a tumour suppressor and negative regulator of translation (Dorrello et al., 2006). Pathological conditions including diabetes, tuberous sclerosis and cancer are associated with deregulation of S6K signaling, emphasizing the importance of obtaining a detailed understanding of its regulation and function (Pende et al., 2000; Shamji et al., 2003; Um et al., 2004). To date, no S6K2-specific substrates have been reported, although S6K2 has been implicated in the increased chemoresistance of small cell lung cancer cells exposed to FGF-2, a function not shared by S6K1 (Pardo et al., 2006).

Similar to other AGC kinases, the activation of S6K1 and 2 is associated with the phosphorylation of multiple conserved serine and threonine residues. The mitogen-stimulated phosphorylation of several S/T-P sites in the auto-inhibitory pseudosubstrate domain C-terminal to the kinase domain, induces a conformational change, unmasking two sites, T252 in the kinase activation loop and T412 (T241 and T401 in S6K2) in the hydrophobic motif, that are essen-

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tial for activation (Pullen and Thomas, 1997). Phosphorylation of T412 by the mammalian target of rapamycin (mTOR), acting within the rapamycin-sensitive TORC1 complex enables binding of the phosphoinositide dependent kinase 1 (PDK1), which phosphorylates T252, rendering S6K1 fully active. In addition, S6K1 is phosphorylated on serine 40 by CK2, which regulates its export from the nucleus (Panasyuk et al., 2006). The subcellular localization of S6K2 is regulated by PKC-mediated phosphorylation of S486, which lies in a nuclear localization sequence (NLS) at the C-terminus (Valovka et al., 2003). Upon growth factor stimulation of cells, a pool of S6Ks is also recruited to receptor tyrosine kinases and becomes tyrosine phosphorylated (Rebholz et al., 2006). Recently, we have also shown that S6Ks are modified by ubiquitination, which targets them to proteasomal degradation (Wang et al., 2008).

In the present study, we identified novel growth factor-inducible interactions between S6Ks and the histone acetyltransferases (HATs), p300/CBP (CREB binding protein) and PCAF. It does not appear that these HATs are substrates for the kinase activities of S6K1 or S6K2 but rather that S6Ks are acetylated, both *in vitro* and *in vivo*. To our knowledge, this is the first direct evidence for acetylation of S6Ks, demonstrating that in addition to multiple phosphorylation and ubiquitination events, S6Ks are regulated by lysine acetylation, a modification that has been shown to play an important role in the regulation of a growing number of proteins, in addition to histones (Kouzarides, 2000).

2. Materials and methods

2.1. Plasmids and recombinant proteins

The Glu-Glu (EE)-tagged expression constructs and recombinant purified S6K1 and S6K2 proteins used in this study have all been described previously (Valovka et al., 2003). Gal4-tagged p300 in the pVR1012 vector (Vical) was from N. Perkins (University of Dundee, Dundee, UK). The p300 fragments were produced by PCR exactly as described in (Hasan et al., 2001a) and cloned into pGEX4T2 to produce GST-fusion proteins in *E. coli*. Recombinant GST-tagged p300-HAT domain was purchased from Upstate Biotechnology and recombinant purified Flag-PCAF was produced as described previously (Itoh et al., 2000).

2.2. Antibodies

The anti-EE monoclonal antibody was a kind gift from Dr. Julian Downward (Cancer Research UK London Research Institute). The anti-S6K1 and anti-S6K2 antibodies have been described previously (Valovka et al., 2003). PCAF and Gal-4 DBD antibodies were from Santa Cruz Biotechnology, rabbit polyclonal anti-acetyl-lysine antibodies were from Cell Signaling Technology and Upstate Biotechnology and were mixed together (each at a dilution of 1:1000) for the detection of acetylated S6Ks by Western blotting. The p300 monoclonal antibody was from Upstate. Phospho-rpS6 (S240/244) and phospho-S6K (T412) antibodies were from Cell Signaling Technology.

2.3. Cell lines and transfections

Human embryonic kidney 293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and MCF-7 cells were grown in DMEM/F-12 medium. All media was supplemented with 10% foetal calf serum, L-glutamine and antibiotics at 10% CO_2/37 °C. Two hundred and ninety-three cells were transfected using either Lipofectamine 2000 (Invitrogen) or ExGen 500 (Fermentas Life Sciences) according to the manufacturer's instructions.

2.4. Western blotting, immunoprecipitations and protein–protein interaction assays

Cell lysates were prepared by scraping into ice-cold lysis buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 1% Triton X-100, 10% glycerol, 10 mM sodium butyrate, 2 mM sodium orthovanadate, complete EDTA-free protease inhibitor cocktail (Roche)). For Western blotting of whole cell lysates, protein concentrations were determined by Bradford assay using the Coomassie protein reagent (Pierce) and absorbance measurements were taken on a Cary spectrophotometer at 595 nm using plastic 1 ml cuvettes prior to SDS-PAGE. For immunoprecipitations, antibodies were added to equal quantities of protein extracts and incubated on a rotating wheel for at least 2 h at 4 °C, followed by incubation with 30 μl of 50% protein-A or G-sepharose suspension (Amersham) for 1 h. Immune complexes were washed 4 times with ice-cold lysis buffer. For purification of S6Ks prior to acetyl-lysine blot or purification of p300 for *in vitro* acetylation assays the last two washes were conducted with lysis buffer containing 500 mM NaCl. Samples were boiled in 2× SDS-PAGE sample buffer for Western blotting or used for *in vitro* assays as described. To assess binding of recombinant S6K2 to GST-p300 fragments, GST-fusion proteins were coupled to glutathione sepharose and incubated for 1 h at 4 °C with 500 ng purified recombinant S6K2 in binding buffer (10 mM Tris–HCl (pH7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA, 1 mM DTT) on a rotating wheel. Beads were washed four times in binding buffer and bound proteins were resolved by SDS-PAGE followed by Western blotting. For the detection of endogenous S6K1 acetylation, HEK 293 or MCF7 cells were grown to 60% confluence and then treated with 1 μM trichostatin-A (TSA) or an ethanol control for 12 h. Cell lysates were pre-cleared with protein-A sepharose before endogenous S6K1 was immunoprecipitated using anti-S6K1 monoclonal antibodies. 5 mg of total cell protein was used in each sample. Acetylation was detected using an anti-pan-acetyl antibody (Upstate).

2.5. In vitro protein acetylation assays

In vitro acetylation assays were carried out using either Gal4-p300 immunoprecipitated from transfected 293 cells or with 100 ng of recombinant p300-HAT domain or GST-wtPCAF (full length). Reactions were performed in 30 μl of reaction buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 10 mM Sodium butyrate) with 2 μg recombinant protein substrate, 1 pmol acetyl-coenzyme A. AcCoA was either ^{14}C-labelled (50 mCi/mmol, Amersham) or unlabelled. In cases where proteins were acetylated with ^{14}C-AcCoA, reaction products were resolved by SDS-PAGE and exposed to a phosphorimager screen (Biorad) or X-ray film following treatment with fluorography enhancing solution (Amplify, Amersham).

2.6. ^{35}S-Methionine/cysteine pulse-chase assay

HEK 293 cells were transfected with pcDNA3.1/EE-S6K2 plasmid in 60 mm dishes as described and grown for 36 h prior to labelling. Medium was removed from the cells and replaced with DMEM lacking methionine and cysteine. Cells were incubated for 1 h and the medium was replaced with Met/Cys-free DMEM containing Pro-mix ^{35}S-labelled methionine and cysteine (Amersham, 80 μCi/60 mm dish). Cells were incubated for 2 h before removal of the labelling medium and two washes with normal DMEM. The cells were then incubated in the presence of DMEM containing a 10-fold excess of unlabelled methionine and cysteine for varying time periods. At each time point (0, 3, 10 and 20 h), cells were washed in ice-cold PBS and snap-frozen. Cells were lysed as described for