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cAMP-elevation mediated by β-adrenergic stimulation inhibits salt-inducible kinase (SIK) 3 activity in adipocytes

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ABSTRACT

Salt-inducible kinase (SIK) 3 is a virtually unstudied, ubiquitously expressed serine/threonine kinase, belonging to the AMP-activated protein kinase (AMPK)-related family of kinases, all of which are regulated by LKB1 phosphorylation of a threonine residue in their activation (T)-loops. Findings in adrenal cells have revealed a role for cAMP in the regulation of SIK1, and recent findings suggest that insulin can regulate an SIK isoform in Drosophila. As cAMP has important functions in adipocytes, mainly in the regulation of lipolysis, we have evaluated a potential role for cAMP, as well as for insulin, in the regulation of SIK3 in these cells. We establish that raised cAMP levels in response to forskolin and the β-adrenergic receptor agonist CL 316,243 induce a phosphorylation of SIK3 in HEK293 cells and primary adipocytes. This phosphorylation coincides with increased 14-3-3 binding to SIK3 in these cell types. Our findings also show that cAMP-elevation results in reduced SIK3 activity in adipocytes. Phosphopeptide mapping and site-directed mutagenesis reveal that the cAMP-mediated regulation of SIK3 appears to depend on three residues, T469, S551 and S674, that all contribute to some extent to the cAMP-induced phosphorylation and 14-3-3-binding. As the cAMP-induced regulation can be reversed with the protein kinase A (PKA) inhibitor H89, and a role for other candidate kinases, including PKB and RSK, could be excluded, we believe that PKA is the kinase responsible for SIK3 regulation in response to elevated cAMP levels. Our findings of cAMP-mediated regulation of SIK3 suggest that SIK3 may mediate some of the effects of this important second messenger in adipocytes.

1. Introduction

Salt-inducible kinase (SIK) 3, one of three SIK isoforms, is a member of the AMP-activated protein kinase (AMPK) subfamily of serine/threonine kinases, all of which share homology with AMPK in their kinase domains. The activity of AMPK and most of its related kinases, is regulated by phosphorylation of a specific threonine residue in their activation (T)-loop, a phosphorylation mediated by the upstream kinase LKB1 [1–4].

SIK1 was discovered in the adrenal glands of rats fed a high-salt diet [5] and subsequent homology searches revealed two additional isoforms, SIK2 and SIK3 [6]. While SIK1 appears to participate in steroidogenesis [7] and SIK2 has mainly been implicated in inhibition of gluconeogenesis in the liver [8], SIK3 is the least investigated of the SIK isoforms, its function and regulation largely unstudied. Recent findings in Drosophila, suggest that an SIK isoform is activated by protein kinase B (PKB) in response to insulin, resulting in regulation of energy balance in the fly [9]. Previous findings in Drosophila suggest a role for SIK3 in spindle pole formation [10], a result corroborated in studies of ovarian cancer cell lines, where overexpression of SIK3 resulted in promotion of cell cycle progression [11]. The recent generation of an SIK3 knock-out mouse model revealed that SIK3 is essential for chondrocyte hypertrophy during skeletal development in mice [12]. Sasagawa et al. also report that SIK3-deficient mice display impaired cholesterol metabolism (unpublished data).

It has been discovered that some 14-3-3 isoforms can bind directly to the T-loop threonine residue (T221 in human SIK3) of SIK1 and SIK3 upon LKB1-phosphorylation, resulting in a change in the subcellular distribution of these kinases [13]. 14-3-3s are scaffolding proteins that homo- and heterodimerize and bind to specific phosphorylated motifs on more than 300 targets, mainly affecting their subcellular localization or interactions with other proteins [14]. In adrenal cells, SIK1 undergoes nucleocytoplasmic shuttling in response to stimuli that raise cAMP levels, a translocation that depends on the phosphorylation of a specific serine residue, S577, which is conserved in SIK3 (S551) [15]. Moreover, we

Abbreviations: AMPK, AMP-activated protein kinase; ATP, adenosine tri-phosphate; cAMP, cyclic adenosine mono-phosphate; CRTC2, CREB regulated transcription coactivator 2; GST, Glutathione-S-Transferase; HEK293, human embryonic kidney 293 cells; HSL, hormone sensitive lipase; IPTG, isopropyl-β-D-thiogalactopyranoside; PKA, protein kinase A; PKB, protein kinase B; RSK, ribosomal S6 kinase; SIK, salt-inducible kinase.

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recently demonstrated that SIK2 is phosphorylated on S358 in response to cAMP in adipocytes, and that this induces its binding to 14-3-3 and relocalization [16]. However, the effect of cAMP-increasing agents, or any other extra-cellular signals, such as insulin/IGF1, on SIK3 in mammalian cells, remains unclear.

In this paper, we investigated potential effects of cAMP as well as insulin on SIK3 phosphorylation, 14-3-3 binding, kinase activity and sub-cellular localization. In order to study the regulation of endogenous SIK3 in a physiologically relevant mammalian cell model, we employed primary rat adipocytes, the function of which is under critical control by both cAMP and insulin [17].

We demonstrate that increased cAMP levels induce a PKA-dependent phosphorylation of SIK3, which correlates with an increase in 14-3-3 binding to sites other than the T-loop T221. Moreover, we demonstrate that this phosphorylation correlates with an inhibition of SIK3 kinase activity. Insulin/IGF1 did however not affect SIK3 phosphorylation or its binding to 14-3-3.

2. Materials and methods

2.1. Materials

Human embryonic kidney (HEK) 293 cells were kindly provided by Dr Kei Sakamoto (University of Dundee, UK) and Sprague–Dawley rats were obtained from Charles River laboratories. Dulbecco's Modified Eagle Medium (DMEM), trypsin/EDTA, forskolin, CL 316,243, pre-coupled HA-agarose, isopropyl-β-D-thiogalactopyranoside (IPTG), glutathione, fish skin gelatine and ampicillin were all obtained from Sigma Aldrich. Fetal Calf serum (FCS), human recombinant epidermal growth factor (EGF), pre-cast Novex SDS Polyacrylamide Bis-Tris gels, dithiothreitol (DTT), lauryl dodecyl sulfate (LDS) sample buffer and Hoechst nuclear stain were all purchased from Invitrogen (Carlsbad, USA). BL21 and DH5α-strains of *Escherichia coli* were kindly provided by Maria Deak at the Division of Signal Transduction Therapy (DSTT) (University of Dundee, UK). Glutathione-S-Transferase (GST)-Sepharose and Protein-G-Sepharose were purchased from GE Healthcare Biosciences (Uppsala, Sweden), complete protease-inhibitor tablets from Roche (Mannheim, Germany), polyethylenimine (PEI) was obtained from Polysciences (Eppelheim, Germany), Hydromount from National Diagnostics (Georgia, USA) and HiSpeed Maxi Kit from Qiagen. P81 phosphocellulose cation-exchange paper was purchased from Whatman (Dassel, Germany), ^{32}Pγ-ATP from Perkin Elmer (Boston, USA), Sakamototide peptide was synthesized by Pepceuticals (Enderby, UK) and AMARA peptide was synthesized by Dr. Graham Bloomberg (University of Bristol, UK). Enhanced Chemiluminescence SuperSignal Reagent, cover slips and slides were purchased from Thermo Scientific (Schwerte, Germany). H89 was purchased from Biomol and IGF-1 from Tocris Bioscience (Bristol, UK). MK2206, PD 0325901 and full-length CREB regulated transcription coactivator 2 (CTRTC2) were kindly provided by the Division of Signal Transduction Therapy (University of Dundee, UK). pCMV5 and pEGFP-C1 cDNA constructs encoding wild type, T221A and kinase inactive (D206A) versions of an HA-tagged human SIK3 sequence obtained from www.kinase.com, were kindly provided by Dario Alessi, University of Dundee, and all other SIK3 mutants were generated by DNA-Cloning-Service (Hamburg, Germany). pGEX vectors encoding GST-tagged human 14-3-3ζ were kindly provided by Professor Carol Mackintosh (University of Dundee, UK). Insulin was supplied by Novo Nordisk (Copenhagen, Denmark).

2.2. Antibodies

The anti-SIK3 and anti-SIK2 C-terminal antibodies used for immunoprecipitation and western blotting of SIK3 and SIK2 respectively, were raised in rabbit against peptides corresponding to residues 1349–1369 (TDILLSYKHPEVSFSMEQAGV) of human SIK3 and residues 906–926 (LFDCEMLDAVDPQHNGYVLVN) of human SIK2 and affinity-purified by Innovagen (Lund, Sweden). The antibody used for immunoprecipitation and kinase assay of AMPKα1 was a kind gift from Professor D. Grahame Hardie (University of Dundee). The following total protein antibodies were used for Western Blotting: anti-HA and anti-GAPDH antibodies were obtained from Sigma Aldrich, anti-AMPK antibody was from Cell Signaling Technology and anti-14-3-3 antibody from Santa Cruz Biotechnology. The following phosphospecific antibodies were used for Western Blotting: anti-phospho HSL S563, anti-phospho PKA consensus, anti-phospho PKB consensus, anti-phospho AMPK T172 and anti-phospho ERK T42/44 were all purchased from Cell Signaling Technologies. Anti-phospho PKB S473 antibody, Alexa Fluor 594 anti-mouse antibody and horseradish-peroxidase (HRP)-conjugated secondary anti-rabbit antibody were all purchased from Invitrogen (Carlsbad, USA). HRP-conjugated secondary anti-mouse antibody was purchased from GE Healthcare.

2.3. Isolation and stimulation of primary rat adipocytes

Primary rat adipocytes were isolated from epididymal adipose tissue of male 36–38 day old Sprague–Dawley rats [18], under a protocol approved by the ethical review committee at Lund University (approval no. M212-09). Adipocytes were suspended in Krebs-Ringer medium containing 25 mM HEPES pH 7.4, 200 nM adenosine, 2 mM glucose and 1% BSA (Krebs-Ringer medium) and stimulated at 37 °C in a shaking water bath (120 rpm). The cells were subsequently washed twice in Krebs-Ringer medium without BSA and homogenized in 0.5–1 ml of homogenization buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM dithiothreitol (DTT) and complete protease inhibitor (1 tablet/50 ml). The cell homogenates were centrifuged for 5 min at 5000 *g* and the fat layer was removed. 1% NP40 was added to the remaining supernatant, the samples were left on ice for 30 min to solubilize and finally centrifuged for 15 min at 4 °C and 13000 *g*. Protein concentrations were determined according to the Bradford protocol.

2.4. Culture, transfection and stimulation of HEK293 cells

HEK293 cells were cultured in DMEM to 100% confluence in 10 cm dishes and split 1:5 into new plates. Approximately six hours post-seeding, each 10 cm plate was transfected with 5 μg DNA of pCMV5 constructs (amplified in *E. coli* DH5α bacteria according to HiSpeed Qiagen maxi preparation protocol) encoding wild type, kinase inactive D206A, T-loop mutant T221A, S469A, S551A, S674A and T221A/S469A/S551A/S674A HA-SIK3 using the PEI method [19]. Cells were grown in serum-free medium for 16 h before stimulation and harvesting. 36 h post-transfection, cells were stimulated, washed once with PBS and lysed in 500 μl lysis buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM dithiothreitol (DTT), 1% NP40 and complete protease inhibitor (1 tablet/50 ml) (lysis buffer).

2.5. Generation of recombinant 14-3-3 for far western

pGEX vectors encoding human GST-tagged wild type 14-3-3ζ were transformed into *E. coli* BL21 bacteria. One liter cultures were grown at 37 °C and 220 rpm in Luria Broth medium containing 100 μg/ml of ampicillin, until the optic density was 0.8. IPTG was added to a concentration of 100 μM in order to induce protein expression and the bacteria were cultured for an additional 16 h at 26 °C at 220 rpm. The cultures were centrifuged for 20 min at 4 °C and 4000 rpm and the bacterial pellet was resuspended in 10 ml ice-cold lysis buffer containing 1 mM DTT and complete protease inhibitors. After one cycle of freeze thawing, the extracts were sonicated for complete bacterial lysis and then