Biochimica et Biophysica Acta 1853 (2015) 1796–1807

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

GSKIP- and GSK3-mediated anchoring strengthens cAMP/PKA/Drp1 axis signaling in the regulation of mitochondrial elongation

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ARTICLE INFO

Article history:
Received 28 January 2015
Received in revised form 15 April 2015
Accepted 21 April 2015
Available online 25 April 2015

Keywords:
GSKIP
AKAP
GSK3
Drp1
Anchoring
Mitochondrial morphology

ABSTRACT

GSK3β binding of GSKIP affects neurite outgrowth, but the physiological significance of PKA binding to GSKIP remains to be determined. We hypothesized that GSKIP and GSK3β mediate cAMP/PKA/Drp1 axis signaling and modulate mitochondrial morphology by forming a working complex comprising PKA/GSKIP/GSK3β/Drp1. We demonstrated that GSKIP wild-type overexpression increased phosphorylation of Drp1 S637 by 7–8-fold compared to PKA kinase-inactive mutants (V41/L45) and a GSK3β binding-defective mutant (L130) under H_2O_2 and forskolin challenge in HEK293 cells, indicating that not only V41/L45, but also L130 may be involved in Drp1-associated protection of GSKIP. Interestingly, silencing either GSKIP or GSK3β but not GSK3α resulted in a dramatic decrease in Drp1 S637 phosphorylation, revealing that both GSKIP and GSK3β are required in this novel PKA/GSKIP/GSK3β/Drp1 complex. Moreover, overexpressed kinase-dead GSK3β–K85R, which retains the capacity to bind GSKIP, but not K85M which shows total loss of GSKIP-binding, has a higher Drp1 S637 phosphorylation similar to the GSKIP wt overexpression group, indicating that GSK3β recruits Drp1 by anchoring rather than in a kinase role. With further overexpression of either V41/L45P or the L130P GSKIP mutant, the elongated mitochondrial phenotype was lost; however, ectopically expressed Drp1 S637D, a phosphomimetic mutant, but not S637A, a non-phosphorylated mutant, restored the elongated mitochondrial morphology, indicating that Drp1 is a downstream effector of direct PKA signaling and possibly has an indirect GSKIP function involved in the cAMP/PKA/Drp1 signaling axis. Collectively, our data revealed that both GSKIP and GSK3β function as anchoring proteins in the cAMP/PKA/Drp1 signaling axis modulating Drp1 phosphorylation.

1. Introduction

Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase, participates in several signaling pathways, including the protein kinase A (PKA), protein kinase B (PKB), protein kinase C (PKC), and Wingless (Wnt) pathways. This protein plays a multifaceted physiological role in the regulation of cell fate, signal transduction, protein synthesis, glycogen metabolism, mitosis, and apoptosis [1,2]. Two structural homologues, GSK3α and GSK3β, have been described in mammals. Differences in the developmental expression profile of GSK3α and GSK3β imply that the regulation and functions of these 2 proteins are not always identical [3]. GSK3β plays an important role in neuron development by regulating the specification of axons and dendrites [4,5]. Our previous work also showed that GSK3β activity is negatively regulated

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http://dx.doi.org/10.1016/j.bbamcr.2015.04.013
0167-4889/

by GSKIP. Overexpression of GSKIP prohibits retinoic acid (RA)-induced neurite outgrowth in SH-SY5Y, a neuron-like cell line. While GSKIP increases β-catenin nuclear translocation and enhances cyclin D1 expression, it down-regulates N-cadherin expression to promote cell cycle progression in SH-SY5Y cells [6].

Our previous work demonstrated that GSK3β also binds to Drp1 and is involved in regulating mitochondrial morphology [7]. Drp1 is one of the dynamin-related proteins, a large protein with an amino-terminal GTPase domain, middle domain, insert B, and GTPase effector domain (GED) [8]. Drp1 participates in cellular events that affect the shape, size, distribution, remodeling, and maintenance of mitochondria in mammalian cells [9]. Phosphorylation is an important regulator of Drp 1 activity. Interestingly, phosphorylation of Drp 1 at multiple serine residues has opposing functional and morphological effects [7,10–18]. Drp1 S637 was first identified as a phosphorylation site for PKA and as a dephosphorylation site for calcineurin [12–14]. Phosphorylation at Drp1 S637 leads to an elongated mitochondrial morphology. Previous studies have indicated that phosphorylation at Drp1 S637 has beneficial effects in starvation conditions, where mitochondrial are observed to be elongated [19,20] and unite to survive [21] by protecting mitochondria from autophagosomal degradation, permitting mitochondria to maximize energy production and supplying autophagosomal membranes [22]. In addition, GSK3β-mediated phosphorylation of Drp1 S693 is important in controlling mitochondrial morphology under oxidative stress [7]. However, the physiological role of such phosphorylation events remains unclear.

GSKIP is a small AKAP that complexes with PKA and GSK3β and is known to act synergistically with cAMP/PKA signaling to inhibit GSK3β activity [23]. GSKIP also interacts directly with GSK3β, negatively regulating GSK3β signaling [24]. According to our previous findings, GSK3β binding of GSKIP is involved in the control of neurite outgrowth [6]. However, the cellular consequences of PKA binding to GSKIP remain to be determined.

In this study, we tested: 1) Whether GSKIP and GSK3β mediate cAMP/PKA/Drp1 axis signaling and contribute to morphological modulation of mitochondria by forming a working complex comprising PKA, GSKIP, GSK3β, and Drp1; 2) Whether and how PKA regulates Drp1 phosphorylation via the PKA/GSKIP/GSK3β/Drp1 signaling axis while inhibiting GSK3β activity; and 3) Whether GSK3β recruits Drp1 by anchoring or by a kinase role. We concluded that both GSKIP and GSK3β function as anchoring proteins involved in cAMP/PKA/Drp1 axis signaling, modulating Drp1 phosphorylation at Ser637 rather than Ser693. This is the first report of the anchoring role of GSK3β in the PKA/GSKIP/GSK3β/Drp1 signaling axis, potentially offering new insights into the multifaceted role of GSK3β.

2. Methods

2.1. Cell culture, differentiation and treatment

Human neuroblastoma SH-SY5Y, HeLa, and HEK293 cell lines (American Type Culture Collection, Manassas, VA, USA) were used for these experiments. SH-SY5Y cells were cultured in DMEM/F12, and HeLa and HEK293 cells were cultured in DMEM (GIBCO BRL Life Technologies, Invitrogen Corporation, CA, USA). All media were supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were incubated at 37 °C with humidification and 5% CO_2. Cells were cultured in 100-mm diameter dishes and fed once every three days until they reached 70% confluence. To induce neuronal differentiation, SH-SY5Y cells were seeded at 1×10^6 cells/cm^2 in 100-mm culture dishes in DMEM/F12 medium containing 10% FBS. When cells were 40–50% confluent, differentiation was initiated by adding 10 μM RA (Sigma-Aldrich, St. Louis, MO, USA). The cells were kept under these conditions for five days, changing the medium every 2 days. For serum starvation, SH-SY5Y cells were incubated in DMEM/F12 medium without 10% FBS for 24 h. For H_2O_2 treatment, SH-SY5Y, HEK293, and HeLa cell lines were treated with 100 μM H_2O_2 (for SH-SY5Y and HEK293) and/or 500 μM H_2O_2 (for HeLa) with or without 10 μM forskolin for 24 h. After 24 h treatment, the cells were cultured in fresh medium and subjected to further assays (HEK293 and SH-SY5Y, cell viability assays and Western blotting; HeLa cells, immunocytochemistry).

2.2. Cloning and DNA sequencing

To construct GFP-tagged wt and mutant GSKIP, DNA fragments encoding GSKIP were amplified by PCR using Taq polymerase (TaKaRa/Clontech, Madison, WI, USA). The PCR fragments were then inserted into the *BamH*I and *Xho*I sites of the pEGFP (Clontech) vector. Site-directed mutagenesis was used to create the GSKIP L130P (Leucine 130 to Proline) and V41/L45P (Valine 41 and Leucine 45 to Proline) mutants using a Quickchange Lightning kit (GE Healthcare, Sunnyvale, CA, USA) according to the manufacturer's protocol. The nucleotide sequencing reaction was performed with a BigDye Terminator v3.1 Cycling Sequencing kit (Applied Biosystems, Waltham, MA, USA) and the extended products were resolved on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Forster City, CA, USA).

2.3. Transfection and RNA interference

For transient transfections, SH-SY5Y, HeLa, and HEK293 cells were seeded onto glass coverslips at a density of 1×10^5 cells per 12-well plate. pEGFP, pEGFP-GSKIP, pEGFP-GSKIP (L130P), pEGFP-GSKIP (V41/L45P), pEGFP-Drp1 (S637D), or pEGFP-Drp1 (S637A) plasmid DNA (1 μg) was transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h transfection, the SH-SY5Y cells were driven to differentiate by adding 10 μM of RA and incubating for five days. For Western blots and immunofluorescence, SH-SY5Y, HeLa, and HEK293 cells were transfected with DNA (1 μg) and Lipofectamine 2000 (3 μL). After 24 h transfection, the cells were cultured in fresh medium and further assayed. Three different siRNA and scrambled siRNA duplexes were used for RNA interference assays: GSKIP (c14orf129), GSK3α, and GSK3β. For the siRNA-mediated knockdown of GSKIP expression, approximately 1×10^5 or 1×10^6 cells were plated onto 12-well plates or 100 mm dishes and left to grow overnight. The following day, cells were transfected with the siRNA duplex (final concentration, 50 nM) using Lipofectamine 2000.

2.4. Quantitative PCR

cDNA was synthesized from total RNA for each of the studied groups using an ImpronII reverse transcriptase kit (Promega, Madison, WI, USA). All primer pairs with respect to GSKIP, LC3B and GAPDH were designed using a web-based program provided by GeneScript.com. All were assessed and demonstrated: 1) high amplification efficiency (>96%) across a wide range of cDNA dilutions; 2) specific, single products in dissociation curve analysis; and 3) melting temperatures similar to those predicted by oligonucleotide software. Quantitative PCR was performed using Power SYBR Green dye and the ABI Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The difference between the cycle thresholds (dCT) for target gene (GSKIP and LC3B) mRNA expression was calculated by subtracting the geometric mean of the cycle threshold for the reference gene (GAPDH) from the cycle threshold of GSKIP mRNA. Because this dCT represents the $\log^2$-transformed expression ratio of the target transcript to the geometric mean of the reference gene, the relative expression level of target gene mRNA was determined as 2-dCT. The relative target gene expression between groups was shown as fold-increases with standard deviations.