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The origin of GSKIP, a multifaceted regulatory factor in the mammalian Wnt pathway

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ABSTRACT

GSK3β interacting protein (GSKIP) is a naturally occurring negative regulator of GSK3β and retains both the Protein Kinase A Regulatory subunit binding (PKA-RII) domain and GSK3β interacting domain. Of these two domains, we found that PKA-RII is required for forming a working complex comprising PKA/GSKIP/GSK3β/Drp1 to influence phosphorylation of Drp1 Ser637. In this study, bioinformatics and experimental explorations re-analyzing GSKIP's biofunctions suggest that the evolutionarily conserved Domain of Unknown Function (DUF727) is an ancestral prototype of GSKIP in prokaryotes, and acquired the C-terminal GSK3β binding site (tail) in invertebrates except for *Saccharomyces* spp., after which the N-terminal PKA-RII binding region (head) evolved in vertebrates. These two regions mutually influence each other and modulate GSKIP binding to GSK3β in yeast two-hybrid assays and co-immunoprecipitation. Molecular modeling showed that mammalian GSKIP could form a dimer through the L130 residue (GSK3β binding site) rather than V41/L45 residues. In contrast, V41/L45P mutant facilitated a gain-of-function effect on GSKIP dimerization, further influencing binding behavior to GSK3β compared to GSKIP wild-type (wt). The V41/L45 residues are not only responsible for PKA RII binding that controls GSK3β activity, but also affect dimerization of GSKIP monomer, with net results of gain-of-function in GSKIP-GSK3β interaction. In addition to its reported role in modulating Drp1, Ser637 phosphorylation caused mitochondrial elongation; we postulated that GSKIP might be involved in the Wnt signaling pathway as a scavenger to recruit GSK3β away from the β-catenin destruction complex and as a competitor to compete for GSK3β binding, resulting in accumulation of S675 phosphorylated β-catenin.

1. Introduction

GSKIP was initially discovered to be a GSK3β interacting protein using a yeast two-hybrid technique in which the GSK3β D200G mutant was used as bait. Wild-type (wt) GSKIP was only found to interact with kinase-dead GSK3β or truncated GSKIP fragments (C-terminal 105–139), but not wild-type GSK3β [1,2]. These results were consistent with other published findings [3,4]. Functionally expressed wild-type GSKIP prohibits neurite growth in the neuronal-like SH-SY5Y cell line, indicating a possible role of GSKIP in the neuronal system [5]. So far, mammalian GSKIP is suggested to be a cytosolic scaffolding protein retaining the protein kinase A (PKA) RII binding sites at residue V41/L45 and glycogen synthase kinase 3β (GSK3β) binding domain at residue L130 [1–3,5–7]. In addition to being a GSK3β interacting protein, GSKIP is also characterized as having a PKA RII binding site and as a small A-kinase anchoring protein (AKAP) [6,7]. Due to this dual

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binding activity, GSKIP functions as an anchoring protein with GSK3β in the cAMP/PKA/Drp1 signaling axis to modulate Drp1 phosphorylation and provide neuroprotection against oxidative stress to neuron-like cells [8]. More recently, it was suggested that GSKIP interaction with PKA and GSK3β is required for Wnt signaling regulation via a cytoplasmic destruction complex targeting β-catenin for degradation, leading to negative regulation of Wnt signaling [7,9].

Knock-out animal models determined that loss of GSKIP expression cause prenatal death due to a developmental defect in palatal shelf fusion [10]. GSKIP was characterized as one of the predisposing genes in familial myeloproliferative neoplasms (MPN) in gene overexpression studies [11–13]. More recently, it was found that depletion of PPARγ-E2F1 axis signaling caused down-regulation of GSKIP, which may indicate that GSKIP is involved in PPARγ-related angiogenic potential in mature PMVECs [14].

GSKIP is found to be conserved across different species, with the DUF727/GSKIP domain as the core component. The functional significance of GSKIP was demonstrated in several human disorders and animal studies, but the signaling pathway involved remains ambiguous. It is likely that GSKIP plays a context-dependent multifaceted role as a scavenger and a competitor in addition to regulating the Wnt pathway. However, several questions remain 1. The origin and development of GSKIP in invertebrates and vertebrates; 2. Why the PKA RII binding domain only exists in vertebrates; 3. How the PKA RII binding domain is involved in GSKIP interaction with GSK3β; 4. How the GSK3β binding site and PKA RII binding domain of GSKIP affect the Wnt signaling pathway. To answer these questions, bioinformatics, yeast two-hybrid, molecular modeling and co-immunoprecipitation techniques were applied to determine the interplay of PKA RII and the GSK3β binding domain of GSKIP in controlling physiological functions.

2. Materials and methods

2.1. Gene ontology and Uniprot analysis

Based on the ranking of the gene-based association analysis, we performed Gene Ontology (GO, http://geneontology.org) and InterPro [http://www.ebi.ac.uk/interpro/] data mining [15]. We performed Uniprot combined with InterPro (http://www.ebi.sc.uk/interpro/) [15] domain enrichment analyses and compared the results to the NCBI website, based on the ranks of the genes from the strict and extended gene-based tests. Keywords such as GSKIP, DUF727, GSK3β, Clu-1, and AMRC4 were used separately or in combination for retrieving GSKIP-related protein data. Conversion from FlyBase gene identifiers to InterPro was also done using FlyBase version FB2014_04.

2.2. Phylogenetic analysis

Multiple sequences with respect to the PKA binding domain [28–53 amino acid (a.a.)], DUF727 and GSK3β binding domain (116–139 a.a.) of GSKIP orthologues were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). An unrooted phylogenetic tree of 17 selected GSKIP orthologues was also generated using the Neighbor-joining method [16]. To ensure the tree structure from Clustal Omega, full length and fragments spanning from 53 to 139 a.a. residues of GSKIP protein were aligned by the T-coffee method accessed through the T-coffee website interface (http://www.tcoffee.org) [17,18]. The fittest amino acid substitution model was estimated by the MEGA 7 program [19]. A phylogenetic tree was reconstructed with the Bayesian method using the LG + G + I substitution model by BEAST V1.8.4 [20]. The robustness of the phylogenetic trees was statistically evaluated by posterior probability.

2.3. Cloning and DNA sequencing

pACT2-GSKIP and pAS2-1-GSK3β for the yeast two-hybrid assay as well as pEGFP-GSKIP plasmids were constructed as described previously [1]. mCherry-GSK3β and pHA-GSKIP plasmid were constructed separately by inserting PCR fragments into commercial HA- or mCherry-tag expression vectors. GSKIP L130P, V41/L45P and GSK3β S9A, S9D, S9E, K85R, K85M, D200G, V267G, Y288F, 9A/85R, and 9A/D200G mutants were created using the site-directed mutagenesis technique of the Quickchange Lightning kit (GE Healthcare, Sunnyvale, CA, USA). All experimental procedures were carried out following the manufacturer's protocol, and mutated nucleotides were verified by DNA sequencing with an ABI PRISMTM 3730 Genetic Analyzer (Perkin-Elmer).

2.4. Yeast two-hybrid system

Standard techniques were used to carry out yeast two-hybrid screening [21–23] using the MATCHMAKER Two-Hybrid System 2 (Clontech). YRG-2 yeast host cells (MATa ura3–52 his3–200 ade2–101 lys2–801 trp1–901 leu2–3 112 gal4–542 gal80–538 LYS2::UAS_{GAL1}-TATA $_{GAL1}$-HIS3 URA3::$UAS_{GAL4\ 17mers(x3)}$-$TATA_{CYC1}$-lacZ) were purchased from Stratagene and co-transfected with the pAS2-1 and pACT2 plasmids and then selected on G2 plates deficient in tryptophan and leucine, and on G3 plates further deficient in histidine. A positive interaction was determined by growth on G3 plates and a visible blue-color pattern in the colony filter lift assay [22]. For assessing the growth inhibition effect of GSKIP in yeast, YRG-2 yeast cells were co-transfected with pACT2-GSKIP and an empty pAS2-1 vector and then spread on G2 and G3 agar plates. LiCl (10 mM) or H89 (10 μM) was added to the plate to inhibit GSK3β or PKA activity, respectively.

2.5. Co-immunoprecipitation

The HEK293 cell line was maintained in DMEM supplemented with 10% FBS. After transfection with expression vectors, HEK293 cells were washed with phosphate-buffered saline (PBS). The lysate was prepared by adding 1 mL of radioimmune precipitation assay buffer [50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1% deoxycholate, and leupeptin, aprotinin, and 4-(2-aminoethyl) benzenesulfonyl fluoride (10 μg/mL each)] to the cells. Samples were kept on ice for 30 min and then centrifuged at 14,000 rpm for 5 min at 4 °C. Anti-Flag (Sigma) antibody was added to the supernatant, and the mixture was incubated at 4 °C for 1 h. Protein-A/G-agarose beads (30 μL) (Oncogene) were added to the lysate, and the mixture was incubated with shaking for 1 h at 4 °C. The beads were spun down and washed three times with radioimmune precipitation assay buffer. Proteins binding to the beads were eluted by adding 20 μL of 2× electrophoresis sample buffer and analyzed by immunoblotting with an anti-HA antibody (Roche).

2.6. Western blot analysis

The cellular lysate was prepared using RIPA buffer. The protein concentration was determined by the Bio-Rad Protein Assay system. Nuclear extracts were prepared using the Nuclear/Cytosol Fractionation kit (Biovision Research Products, Mountain View, CA) according to the manufacturer's instructions. Total cell lysate or immunoprecipitates in SDS sample buffer were heated to 95 °C for 5 min followed by analysis by 12% SDS-PAGE. The proteins were then transferred to PVDF and incubated for 1 h in blocking buffer (5% nonfat milk in TBS with 0.1% Tween 20). Anti-β-Catenin 33/37/41, Ser675 Ab (Cell Signaling Technology), actin, or HA polyclonal antibody incubations were carried out first in blocking buffer for 1 h at room temperature, and then HRP-conjugated antibody was used as the secondary antibody for an additional 1 h.