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Crystal structure of GSK3β in complex with the flavonoid, morin

Kuglae Kim [a, c], Jeong Seok Cha [a], Jin-Sik Kim [b, d], Jinsook Ahn [b], Nam-Chul Ha [b, **], Hyun-Soo Cho [a, *]

[a] Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea
[b] Department of Agricultural Biotechnology, Center for Food Safety and Toxicology, Center for Food and Bioconvergence, Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 08826, Republic of Korea
[c] Department of Pharmacology, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC 27516, USA
[d] Unit on Structural and Chemical Biology of Membrane Proteins, Cell Biology and Neurobiology Branch, National Institute of Child Health and Human Development, National Institutes of Health, 35A Convent Drive, Bethesda, MD 20892, USA

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ABSTRACT

GSK3β is a key kinase that plays a role in cellular signaling pathways. In Alzheimer's disease (AD), GSK3β has been implicated in hyperphosphorylation of tau proteins in the neuron, which is a hallmark of AD. Morin, a flavonoid that is abundant in nature, was found as an inhibitor of GSK3β that can reduce tau pathology *in vivo* and *in vitro*. In this study, we determined the crystal structure of GSK3β in complex with morin. The structure revealed that morin inhibits GSK3β by binding to the ATP binding pocket. Our findings augment the potential of morin as a functional food to help prevent AD, as well as to provide structural information to develop new therapeutics based on the morin skeleton.

1. Introduction

Glycogen synthase kinase 3β (GSK3β) is a Ser/Thr protein kinase that was originally identified as a key regulator of glycogen metabolism in the insulin signaling pathway. The enzyme is now known to be involved in various biological functions, including the Wnt, Hedgehog, Notch, and inflammation signaling pathways [1,2]. Unlike most kinases, GSK3β has a constitutive activity in its resting state and transduces cellular signaling via inhibition of its activity [3].

In neurons, GSK3β is highly expressed and plays important roles in regulating structural and metabolic plasticity by phosphorylating diverse proteins [4]. Especially, GSK3β phosphorylates tau proteins, which are microtubule-associated proteins abundantly expressed in neurons of the peripheral and central nervous systems [5]. The phosphorylation state of tau regulates the binding ability and stabilization of microtubules. However, the hyperphosphorylation of tau proteins can lead to their detachment from microtubules and formation of filamentous self-aggregates in the neuron [6]. This insoluble deposition of tau proteins in neurons results in a class of diseases collectively known as tauopathies [7]. Tauopathies encompass more than 20 clinicopathological entities, including Alzheimer's disease (AD). β-Amyloid precursor protein (APP), presenilin, and tau exert crucial roles in the pathogenesis of AD. Increased production of amyloid β protein (Aβ), converted from APP by presenilin, results in deposition into plaque in the extracellular region, and leads to the pathogenic hyperphosphorylation of tau by increasing the activity of GSK3β. However, how GSK3β activity is elevated by Aβ has not been fully understood [6].

Morin (3,5,7,2′,4′-pentahydroxyflavone) is a flavonoid with antioxidant properties that can protect cells against damage from oxygen radicals. Morin was originally isolated from mulberry figs and heartwood of old fustic (*Chlorophora tinctoria*), and it is rich in many oriental medicinal herbs [8]; thus, it can be isolated from nature. Morin has diverse physiological effects, including anticancer effects [9,10]. Morin was previously reported to directly inhibit GSK3β, resulting in blocking GSK3β-induced tau phosphorylation *in vitro* and *in vivo* [11]. Furthermore, morin attenuated Aβ-induced tau phosphorylation and cytotoxicity in human neurons [11]. Reductions in tau hyperphosphorylation were observed in hippocampal neurons in a 3xTg-AD mouse model by intraperitoneal administration of morin for 7 days [11]. However,

* Corresponding author.
** Corresponding author.
E-mail addresses: hanc210@snu.ac.kr (N.-C. Ha), hscho8@yonsei.ac.kr (H.-S. Cho).

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it remains unknown how morin inhibits GSK3β at the atomic level. In this study, we present the structural basis for the role of morin by presenting the crystal structure of GSK3β in complex with morin.

2. Materials and methods

2.1. Expression and purification

The expression and purification of the mouse GSK3β kinase domain have been previously described [12]. Mouse GSK3β (residues 14–420) was ligated into the pFASTBAC-HTA vector (Invitrogen, USA) to generate the recombinant baculovirus for the Bacto-Bac baculovirus expression system (Invitrogen). The resulting construct had an appended His6-affinity purification tag and a cleavage site for the TEV protease to GSK3β sequence. According to the manufacturer's instructions, recombinant baculovirus was constructed. The protein was expressed after infection of the virus at a multiplicity of infection of 5 in 2.0×10^6 SF9 cells in CCM3 media (Hyclone, USA). The cells were harvested by centrifugation and stored at −80 °C until use. The cells were thawed in a buffer consisting of 20 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM imidazole, 3 mM β-mercaptoethanol, and 10% glycerol and disrupted by homogenization (100 strokes). The cell lysate was applied to Ni-NTA metal affinity chromatography, and then, the His6-tagged protein was pooled and incubated with the recombinant TEV protease overnight to cleave the His6 tag. After changing the buffer by a desalting column in a buffer containing 20 mM HEPES (pH 7.5), 50 mM NaCl, and 1 mM DTT, Resource S column (GE Healthcare, USA) was applied using a linear gradient of 0.05–1 M NaCl in 20 mM HEPES (pH 7.5). The GSK3β protein was eluted at 150 mM NaCl, and the fractions were pooled. The final protein sample was concentrated to 5 mg/mL using a centrifugal concentrator (Millipore, USA).

2.2. Crystallization

Prior to the crystallization, morin (Sigma, USA) was incubated at 5 mM final concentration in the GSK3β protein sample (5 mg/ml) for 30 min at 4 °C. Co-crystallization of the GSK3β protein with morin was performed using the hanging drop vapor diffusion technique. The plate crystals were observed at 17 °C under a condition optimized for hanging drop experiments by mixing 1.5 μL of the protein sample with the same volume of the reservoir solution containing 18% (v/v) PEG 4000, 100 mM sodium citrate (pH 6.5), and 5% (v/v) 2-propanol.

2.3. Data collection and structural determination

The crystals were briefly soaked in a cryoprotectant solution of the reservoir solution supplemented with 20% ethylene glycol. Diffraction data were collected from single crystals flash-frozen at −173 °C in a nitrogen stream. The dataset was measured to a 2.14 Å resolution using a beamline 5C on a Pohang accelerator laboratory synchrotron with X-rays at a 1.0 Å wavelength [13]. The data were auto-indexed and processed with the HKL suite [14]. The crystals of GSK3β with morin complex belonged to the space group $P2_1$. The crystal structure was solved by molecular replacement using human GSK3β, PDB code 1I09 [15] as the search model in the program MOLREP [16]. Coot was used for the visualization of the electron density maps and the manual rebuilding of the atomic model [17]. The model was refined using the program REFMAC5 [18] at a 2.14 Å resolution.

2.4. Surface plasmon resonance (SPR)

For the SPR experiment, we prepared the His6-tagged GSK3β protein by skipping the TEV cleavage step during purification. The purified His6-tagged GSK3β protein was immobilized in the GLH sensor chip (BIO-RAD) by using the ProteOn™ XPR36 machine. Morin in DMSO was prepared at various concentrations (0, 6.25, 12.5, 25, 50, and 100 μM), and each was passed over the GSK3β-immobilized chip. All data were processed by the 1:1 Langmuir binding model, and the binding kinetics and dissociation constant (kD) were calculated.

3. Results

3.1. Structural determination and overall structure

Recombinant GSK3β produced in the insect cells using the baculovirus expression system was co-crystallized with morin. The crystal structure of the complex was solved by the molecular replacement method using a known structure of human GSK3β [15] as a search model. The asymmetric unit contained four GSK3β protomers. The Ramachandran plot showed that 100% of the non-glycine residues were in favored or allowed regions. The refined model presented good stereochemistry, as evaluated by the program MolProbity [19]. The crystallographic data and refinement statistics are shown in Table 1.

The overall structures of GSK3β was almost identical to the GSK3β structures of apo-form, ADP complex, ADP, and an inhibitory peptide bound complex [20]. Each protomer consisted of the N-terminal domain (N-lobe) and the C-terminal domain (C-lobe) between the nucleotide and substrate binding sites as shown in Fig. 1A. All protomers contained a phosphorylated tyrosine at residue 216 as observed in previous structures [20]. The asymmetric unit could be divided into two dimers, and the root-mean-square deviation (RMSD) value between the two dimers was 2.68 Å between the 637 matched Cα atoms (Fig. 1B). Each dimer consists of two GSK3β molecules that had interactions between the N-lobe and C-lobe (Fig. 1C), which was different from the putative dimeric

Table 1
Data collection and refinement statistics.

	GSK3β morin complex
Data collection	
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	67.6, 134.4, 100.4
β (°)	103.8
Resolution (Å)	40.0–2.14 (2.18–2.14)
R_{merge} (%)	7.6 (34.9)
I/σ_I	38.2 (3.8)
Completeness (%)	97.8 (95.0)
Redundancy	6.0 (4.3)
Refinement	
Resolution (Å)	40.00–2.14
No. reflections	88,879
R_{work}/R_{free} [a] (%)	19.1/22.6
No. atoms	
Protein	10,463
Ligand/ion	68
Water	422
B-factors	
Protein	49.29
Ligand/ion	49.71
Water	43.99
R.M.S deviations	Bond length 0.020
	Bond angle 1.62

The numbers in parentheses are statistics for the highest-resolution shell.
[a] R_{free} was calculated with 5% of the data set.

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