IDENTIFICATION OF AIP AS A GSK-3 BINDING PROTEIN

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ABSTRACT. GSK-3, a well-known serine/threonine kinase is one of the key players controlling numerous cellular and physiological processes such as protein synthesis, cell proliferation, cellular differentiation, apoptosis and microtubule dynamics. Therefore, GSK-3 phosphorylates and regulates the functions of a diverse group of substrates including many transcription factors, components regulating the cell cycles and signaling proteins. However, the mechanisms by which GSK-3 regulates the functions of many substrates specifically and selectively are not known. In order to understand the molecular basis of GSK-3 regulation and specificity, we attempt to search for novel GSK-3 binding proteins using yeast two-hybrid screening. We have identified AIP (Aurora-A Kinase Interacting Protein) as a protein that interacts with GSK-3. AIP has been reported to be a novel negative regulator of Aurora-A kinase where it might down-regulates Aurora-A kinase through proteasome dependent degradation. Our study showed that AIP is able to bind both the homologous forms of GSK-3, GSK-3a and GSK-3b in intact cells. This binding is not affected by SB216763, a specific GSK-3 inhibitor, indicating that the kinase activity of GSK-3 is not required for the interaction. AIP has the consensus motif –S-X-X-X-S– for substrate phosphorylation by GSK-3b and is phosphorylated by GSK-3b in vitro. Our results suggest that AIP is a novel binding partner of GSK-3.

KEYWORDS. GSK-3, Aurora-A kinase, AIP, Protein binding

INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) is a key component occupying the central stage of many cellular and physiologically processes including gene expression, microtubules organization, cell proliferation, insulin action and apoptosis (Jope and Johnson, 2004; Fame and Cohen, 2001).

In human, there are two GSK-3 isoforms, GSK-3a and GSK-3b. These two isoforms display 84% overall identity. The activity of GSK-3b is regulated at multiple levels via phosphorylation, localization and its interactions with a diverse group of proteins. Some of the GSK-3b-binding proteins are Axin, Axil, AKAP220, h-prune, Tau and Bicaudal-D. (Ikeda et al., 1998; Yamamoto et al., 1998; Tanji et al., 2002; Kobayashi et al., 2006; Fumoto et al., 2006). Most of the GSK-3 substrates require pre-phosphorylation by other kinases (priming kinases) for efficient phosphorylation by GSK-3, indicating another level of regulation of this signaling system. In addition, the formation of multi-protein complexes also carefully fine-tunes GSK-3 regulation on each individual substrate. However, the detail mechanisms by which GSK-3 controls the functions of each substrate specifically and selectively are not known. In order to understand the molecular basis of GSK-3 regulation and its specificity, we have carried out yeast two-hybrid screening to search for novel GSK-3 binding protein. In this attempt, we have identified AIP (Aurora-A Kinase Interacting Protein) as a GSK-3 binding partner.
AIP was first identified as an Aurora-A kinase interacting protein that down-regulates this kinase through a proteasome-dependent degradation pathway when expressed ectopically (Kiat et al., 2002, Lim et al., 2007). Aurora-A is a prominent mitotic kinase playing critical roles in centrosome cycle, spindle assembly, chromosome segregation, spindle checkpoint and cytokinesis (Marumoto et al., 2005; Meraldi et al., 2004; Nigg 2001).

MATERIALS AND METHODS

Yeast two-hybrid Screening
Yeast two-hybrid screening was carried out using strain L40 (MATa trpl1 leu2 his3 ade2 LYS2::lexA-HIS3 URA3::-lexA-lacZ). The yeast strain L40 carrying pBTM116HA/GSK-3β was transformed with a rat brain cDNA library constructed in pGAD10. pBTM116HA/GSK-3β directs the expression of a fusion between the DNA-binding domain of LexA and the entire GSK-3β from an ADH promoter. The screening was carried out on SD plate media lacking tryptophan, leucine and histidine as evidenced by transactivation of a LexA–HIS3 reporter gene and histidine prototrophy. His+ colonies were scored for β-galactosidase activity. Plasmids harboring cDNAs were recovered from positive colonies and the nucleotide sequences were determined.

Plasmid constructions and Protein Purification
pRSETC-GSK-3β was constructed as previously described (Ikeda et al., 1998; Hino et al., 2003). Standard recombinant DNA techniques were used to construct the following plasmids: pGFP-AIP and pMal-AIP. Recombinant proteins were expressed and purified from E. coli using standard protocols.

Cell Culture, Transfection and Drug Treatment
HEK293T cells and COS cells were grown under 5% CO₂ at 37°C in HEK293T in DH10 medium and DMEM, respectively, supplemented with 10% fetal calf serum. Transfection of the cultured cell line was carried out using LipofectAMINE (Invitrogen) according to the manufacturer's recommended protocol. Cells were transfected with plasmids at a total concentration of 1mg. For drug treatment experiments, transfected cells were treated with different concentration of SB216763 for 2 hours, prior to cell lysate recovery.

Cell extracts, In vitro Binding, Immunoprecipitation and Western Blotting
Cells from 60 mm diameter dish were washed once with ice-cold PBS buffer and lysed with lysis buffer (25 mM Tris/HCl, pH 8, 50 mM NaCl, 0.5% Triton X-100, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 5 mM phenylmethylsulfonyl fluoride). After 15 min on ice, the lysed cells were centrifuged at 15,000 rpm for 15 min at 4°C. Cell lysates were obtained for subsequent experiments. For transiently expressed proteins, respective antibodies were used for immunoprecipitation for 1 hr at 4°C performed in a rotating wheel, followed by Protein A beads (Bio-Rad Laboratories) for another 1 hour. Immune complexes were spun down, washed and then boiled in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Whatman Schleicher and Schuell). For in vitro binding assay, amylose resin was used and performed in a rotating wheel for 1 hour at 4°C. Antibodies used for Western blot analyses in this study include monoclonal antibodies: anti-GFP (MBL), anti-GSK-3β (BD Bioscience), anti-GSK-3α/β (BD Bioscience), anti-His₆ (Clontech) and anti-MBP (Clontech).
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**In vitro phosphorylation assay**

*In vitro* phosphorylation assays were carried out at 30°C for 30 min in buffer containing 50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 50 μM ATP and 50 μCi of [γ-³²P]ATP. The reactions were stop by SDS-PAGE loading dye. Proteins were then resolved by SDS-PAGE and autoradiographed.

**RESULTS AND DISCUSSION**

In our yeast two-hybrid screening using GSK-3β as bait, we have identified AIP (Aurora-A Kinase Interacting Protein) as a positive clone. We then cloned the full length cDNA of AIP that encodes a 199 amino acid polypeptide with a predicted molecular mass of 22 kDa. Computer-assisted analyses showed the C-terminus of this protein is highly hydrophobic and contained tandem bipartite nuclear localization signal as indicated in Figure 1a, suggesting AIP is a nuclear protein. When we transfected pGFP-AIP into COS cells, it was found exclusively overexpressed in the nucleus (Figure 1b).

![Figure 1](image1.png)

**Figure 1.** (a) Deduced amino acid sequence of full-length AIP. The possible GSK-3β phosphorylation site SXXXS is boxed. The tandem bipartite nuclear localization signal is underlined. (b) GFP-AIP is transiently overexpressed in the nucleus of COS cells.

![Figure 2](image2.png)

**Figure 2.** Recombinant GFP-tagged-AIP protein binds to endogenous GSK-3β in HEK293T cells.
In order to verify GSK-3 and AIP interaction in mammalian cells, we transfected HEK293T cells with pGFP-AIP or empty vector as control using Lipofectamine 2000. Cell lysates were collected and used for immunoprecipitation with mouse GSK-3 monoclonal antibody. The immunoprecipitates were separated by PAGE and blotted onto nitrocellulose membrane probed with anti-GFP antibody. Figure 2 showed the GFP-AIP was co-immunoprecipitated together with the endogenous GSK-3β in the cell lysate. The same blot was re-probed with anti-GSK-3β antibody for verification. A reciprocal experiment was performed where cell lysate from HEK293T cells overexpressing GFP-tagged AIP were immunoprecipitated with anti-GFP antibody and immunoblotted with anti-GSK-3α/β antibody, both GSK-3 isoforms were observed (Figure 3). These results confirm that AIP interacts with GSK-3 and it is able to bind both GSK-3α and β isoforms in intact cells.

To further confirm AIP-GSK-3 interaction, binding assay using bacterially expressed recombinant proteins was performed. Maltose Binding Protein (MBP)-tagged AIP and His-tagged GSK-3β were independently expressed and purified from E. coli. Amylose resin bound MBP-AIP was incubated with increased amount of His-GSK-3β. Subsequently, the proteins were probed with anti-His and anti-MBP antibodies as shown in Figure 4. The binding affinity of His-GSK-3β to MBP-AIP increased as the amount of His-GSK-3β increased. This was not observed when MBP was used in the binding assay, indicating in vitro AIP-GSK-3β binding is specific. Taken together, all these data demonstrate that AIP is able to bind GSK-3β both in vivo and in vitro.

Next, we tested whether GSK-3 activity is required for its interaction with AIP. For this purpose, HEK293T cells transiently expressing GFP-AIP were treated with SB216763 at different concentration as indicated in Figure 5. SB216763 is a specific inhibitor for GSK-3 with nanomolar potency. GFP-AIP was co-precipitated with endogenous GSK-3β from both treated and non-treated cell lysates. AIP-GSK-3 binding is not affected by SB216763 treatment and the amount of GFP-AIP precipitated is not affected by SB216763 concentrations. The kinase activity of GSK-3 is one level of regulation on its multiple binding partners where it is not required for complex formation with some of its substrate such as AKAP220 (Tanji et al, 2002) while it is necessary for Axin (Ikeda et al, 1998) and prune (Kobayashi et al, 2006).

Figure 3. AIP binds to both GSK-3α and GSK-3β isoforms in HEK293T cells.
Figure 4. *In vitro* binding assay of bacterially expressed GSK-3β and AIP.

GSK-3 is known to phosphorylate its substrate with consensus sequence –S-X-X-X-S (Plyte et al., 1992). Amino acids sequence analysis of AIP revealed the consensus motif –S-X-X-X-S as boxed in Figure 1a, indicating AIP might be a target for GSK-3 phosphorylation. In order to determine whether AIP is a substrate for GSK-3β, *in vitro* phosphorylation assay using bacterially expressed recombinant GSK-3β and AIP proteins was carried out. In Figure 6a, lane 1 revealed the ability of His-tagged GSK-3β protein to autophosphorylate as expected and MBP is not a substrate for GSK-3β. Lane 2 showed that AIP is unable to autophosphorylate. However, a phosphorylated band of AIP was observed in lane 3 when GSK-3β was present. These results suggest that AIP is phosphorylated by GSK-3β *in vitro* and this phosphorylation is both in a time (Figure 6b) and dose (Figure 6c) dependent manner.

Figure 5. Binding of GSK-3β and AIP is not affected by SB216763 treatment.
We have identified and confirmed Aurora-A Interacting Protein (AIP) as a novel binding partner of GSK-3b. AIP is able to associate with both a and b isoforms of GSK-3 in intact cells. The binding of AIP and GSK-3b is not regulated by its activity because treatment with GSK-3 inhibitor in the binding assay does not affect the binding affinity. In addition, we have demonstrated that AIP is a substrate for GSK-3b using in vitro phosphorylation assay. Nevertheless, the molecular mechanism of AIP and GSK-3b association and its function are yet to be established.

REFERENCES


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