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 poliferation, 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 i sphosphorylated 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 (*MAT***a** *trp1 leu2 his3 a*de*2 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% CO_2 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-His_6 (Clontech) and anti-MBP (Clontech).

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 $MgCl_2$, 1 mM dithiothreitol (DTT), 50 μM ATP and 50 μCi of [γ-^{32}P]ATP. The reactions were stop by SDS-PAGE loading dye. Proteins were then resolved by SDS-PAGE and autoradiographed.

RESULTS AND DICUSSION

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).

(a)

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M L L G R L T S Q L L R A V P W A G G R P P W P V S G V L G   30
S R V C G P L Y S T S P A G P G R A A S L P R K G A Q L E L   60
E E M L V P R K M S V S P L E S W L T A R C F L P R L D T G   90
T A G T V A P P Q S Y Q C P P S Q I G E G A E Q G D E G V A  120
D A P Q I Q C K N V L K I R R R K M N H H K Y R K L V K K T  150
R F L R R K V Q E G R L R R K Q I K F E K D L R R I W L K A  180
G L K E A P E G W Q T P K I Y L R G K                      199
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(b)

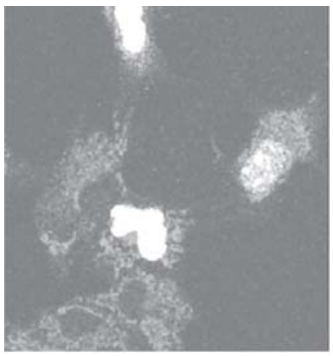

Figure 1. (a) Deduced amino acid sequence of full-length AIP. The possible GSK-3β phosphorylation site SXXXS is boxed. The tandem bipartite nuclear localization signal is underlined. (b) GFP-AIP is transiently overexpressed in the nucleus of COS cells.

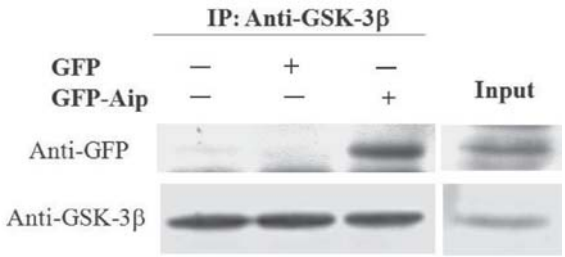

Figure 2. Recombinant GFP-tagged-AIP protein binds to endogenous GSK-3β in HEK293T cells.

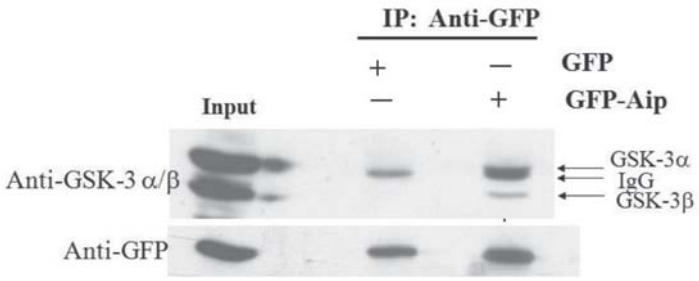

Figure 3. AIP binds to both GSK-3α and GSK-3β isoforms in HEK293T cells.

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-3β 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, *in vitro* 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 acitivity 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).

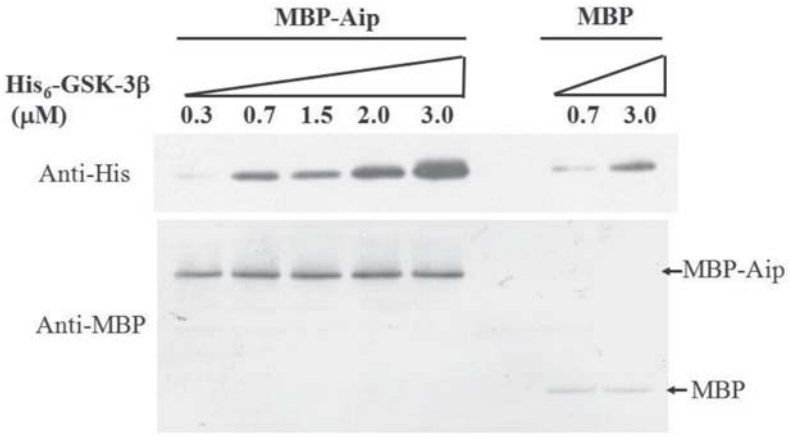

Figure 4. *In vitro* binding assay of bacterially expressed GSK-3b and AIP.

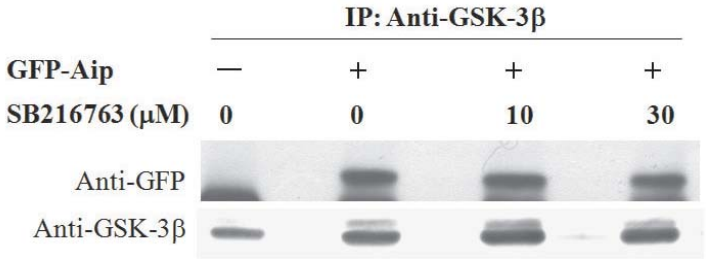

Figure 5. Binding of GSK-3b and AIP is not affected by SB216763 treatment.

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 phosphoyrlation. 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-3βprotein 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.

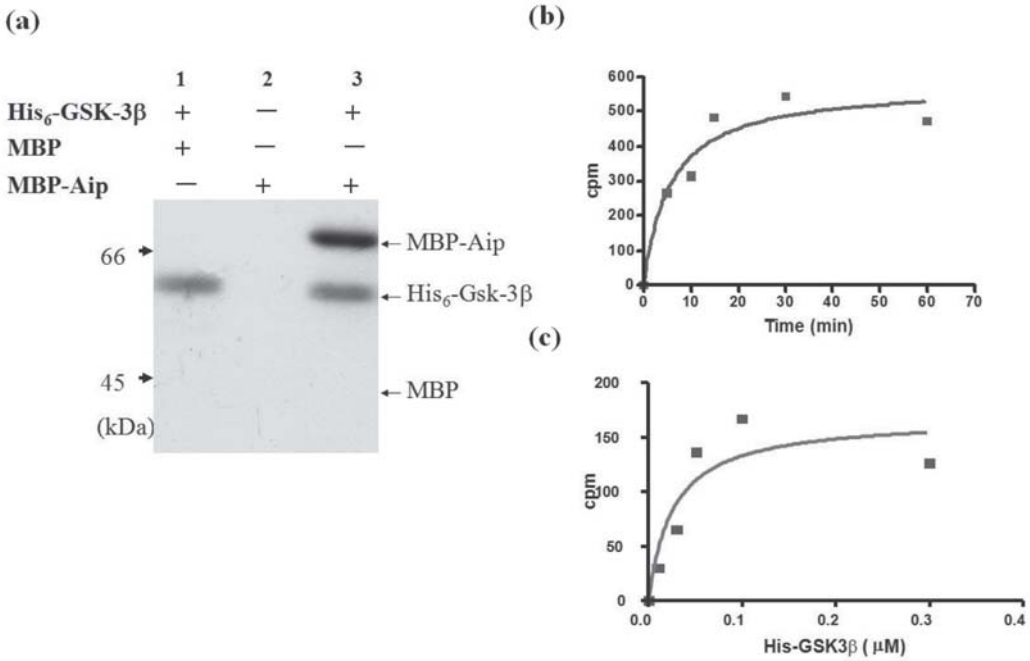

Figure 6. (a) Autoradiograph of *in vitro* phosphorylation assay using recombinant GSK-3β and AIP proteins (b) Time dependent phosphoryalation of AIP by GSK-3β (c) Dose dependent phosphorylation of AIP by GSK-3β.

CONCLUSIONS

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.

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