GSKIP Is Homologous to the Axin GSK3β Interaction Domain and Functions as a Negative Regulator of GSK3β[†]

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ABSTRACT: Although prominent FRAT/GBP exhibits a limited degree of homology to Axin, the binding sites on GSK3 for FRAT/GBP and Axin may overlap to prevent the effect of FRAT/GBP in stabilizing β-catenin in the Wnt pathway. Using a yeast two-hybrid screen, we identified a novel protein, GSK3β interaction protein (GSKIP), which binds to GSK3β. We have defined a 25-amino acid region in the C-terminus of GSKIP that is highly similar to the GSK3β interaction domain (GID) of Axin. Using an in vitro kinase assay, our results indicate that GSKIP is a good GSK3β substrate, and both the full-length protein and a C-terminal fragment of GSKIP can block phosphorylation of primed and nonprimed substrates in different fashions. Similar to Axin $GID_{381-405}$ and FRATtide, synthesized GSKIPtide is also shown to compete with and/or block the phosphorylation of Axin and β-catenin by GSK3β. Furthermore, our data indicate that overexpression of GSKIP induces β-catenin accumulation in the cytoplasm and nucleus as visualized by immunofluorescence. A functional assay also demonstrates that GSKIP-transfected cells have a significant effect on the transactivity of Tcf-4. Collectively, we define GSKIP as a naturally occurring protein that is homologous with the GSK3β interaction domain of Axin and is able to negatively regulate GSK3β of the Wnt signaling pathway.

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase that was first identified by its ability to phosphorylate glycogen synthase and regulate glycogen metabolism (*1*). Mammalian GSK3 has been cloned in two closely related isoforms, GSK3α and GSK3β,[1] which are 98% identical in their catalytic domains (*2*). GSK3β acts as a key enzyme and, when dysregulated, seems to be involved in prevalent human diseases such as diabetes, cancer, Alzheimer's disease, and bipolar disorder (*3*). Regulation of GSK3β occurs primarily via impacts at various cellular levels, including phosphorylation, intracellular distribution, and protein−protein interaction (*4*).

It is known that stimulation of cells with insulin causes inactivation of GSK3β through the phosphoinositide 3-kinase (PI3-kinase) pathway. PI3-kinase can induce activation of protein kinase B (PKB, also called Akt), resulting in PKB phosphorylation of GSK3β at Ser9, which inhibits GSK3β activity (*5*−*7*). Recent results also indicate that the phosphorylation of Tyr216 of GSK3β is important for activity, because dephosphorylation of this amino acid decreases enzyme activity. Conversely, phosphorylation of Tyr216 enhances the activity and stability of this enzyme (*8*, *9*). Recent studies have indicated that intracellular localization mechanisms are also involved in the control of GSK3β activity. For example, GSK3β is found predominantly localized to the cytoplasm, but it also shows high levels of activity in nucleus, mitochondria (*10*), centrosomes, and spindle poles (*11*).

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[1] Abbreviations: GSK3, glycogen synthase kinase-3β; GSKIP, GSK3β interaction protein; GID, GSK3β interaction domain; PI3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; APC, adenomatous polyposis coli; Dvl, Dishevelled; FRAT, frequently rearranged in advanced T-cell lymphoma; GBP, GSK3β binding protein; LRP5, lipoprotein receptor-related protein 5; 3-AT, 3-aminotriazole; GST, glutathione *S*-transferase; GS, glycogen synthase.

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GSK3β has also been reported to be regulated by GSK3β-binding proteins, the best examples of which are found in the Wnt signaling pathway (*4*). The Wnt proteins (the name is derived from mouse Int-1 and *Drosophila* Wingless) are a large family of signaling molecules that have well-established roles in regulating cell fate, differentiation, proliferation, and potential tumor formation (*12*, *13*). It is well-documented that in the absence of Wnt signaling, β-catenin becomes associated in a complex that includes GSK3β, Axin, and adenomatous polyposis coli tumor suppressor protein (APC). Phosphorylation of β-catenin by GSK3β in the complex results in its ubiquitination and subsequent degradation by proteosomes (*14*−*17*). Conversely, with the Wnt signal, the complex is disrupted when Dishevelled (Dvl) is activated, and this, together with GSK3β binding protein (GBP), or the mammalian homologue FRAT, causes GSK3β to move away from Axin and β-catenin (*18*, *19*). Recent results have also shown that the interaction between FRAT/GBP and low-density lipoprotein receptor-related protein 5 (LRP5) mediates the recruitment of Axin, GSK3β, and FRAT/GBP to the membrane, leading to an activation of the Wnt canonical pathway (*20*). When the level of phosphorylation of β-catenin is decreased, this results in β-catenin accumulation and the protein acts as a cotranscription factor (*21*). Furthermore, FRAT/GBP can inhibit GSK3β activity in vivo and mediates its effects on dorsal development in *Xenopus* embryos (*22*). FRAT/GBP also can regulate GSK3β nuclear export (*23*). Significantly, recent results have shown that two peptides derived from FRAT/GBP and the GSK3β interaction domain (GID) of Axin can inhibit GSK3β activity (*24*−*27*), and thereby, the activity of GSK3β can be regulated through its interaction proteins.

Since few naturally occurring GSK3β interaction proteins appear to work by acting as inhibitors that target GSK3β activity, we made an effort to survey possible GSK3β-interacting proteins from a human testis cDNA library (Clontech) using the yeast two-hybrid system. We have identified a novel GSK3β binding protein, designated GSKIP (GSK3β interaction protein, GenBank entry NP_057556), the C-terminal region of which possesses a 25-amino acid region similar to $GID_{381-405}$ of Axin, and found that this region is required for GSK3β binding. Our results suggest that GSKIP and GSKIPtide (a peptide corresponding to residues 115−139 of GSKIP) may act as inhibitors of GSK3β, and this may also be involved in the GSK3β−Axin−β-catenin complex of the Wnt signaling pathway.

MATERIALS AND METHODS

Yeast Two-Hybrid System. Standard techniques were used to carry out yeast two-hybrid screening (*28*−*30*). Briefly, GSK3β was cloned in frame with the Gal4 DNA binding domain in the pAS2-1 vector (MATCHMAKER Two-Hybrid System 2, Clontech) to yield the pAS2-1-GSK3β bait plasmid. A human testis cDNA library was screened by cotransforming yeast YRG-2 (Stratagene) with the pAS2-1-GSK3β bait plasmid DNA and human adult testis library plasmid DNA (Clontech). Positive clones have the ability to grow on Trp, Leu, His dropout medium supplemented with 3-aminotriazole (3-AT, an inhibitor of HIS3), and they turn blue in a β-galactosidase filter assay.

Cloning and DNA Sequencing. To construct the pACT2-GSKIP plasmid for the yeast two-hybrid working assay, DNA fragments encoding GSKIP were amplified by PCR using the Taq polymerase (TaKaRa). The PCR fragments were then inserted into the *Bam*HI and *Xho*I sites of the pACT2 (Clontech) vector. The C-terminus (amino acids 109−139 and 115−139) and N-terminus (amino acids 1−108) of GSKIP were amplified by PCR. These amplified fragments were digested by *Bam*HI and *Xho*I, and they were also introduced into the pACT2 vector. Full-length GSKIP was inserted into the pcDNA vector using the *Bam*HI and *Xho*I restriction sites. Full-length GSKIP was also inserted into the pIRES-hyg2 vector using *Bam*HI sites. Site-directed mutagenesis experiments to create the GSKIP mutants (leucine 130 to proline) were carried out according to the manufacturer's protocol (Stratagene). The nucleotide sequencing was performed with an ABI PRISMTM 3730 Genetic Analyzer (Perkin-Elmer).

Northern Blot Analysis. A human Northern blot containing poly(A^+)-RNAs from adult tissues, including the heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, and from human fetal tissues, including the brain, lung, liver, and kidney, was purchased from Clontech and hybridized for 16−18 h at 68 °C in formamide, 10× Denhardt's solution, 5× buffer A [0.75 M sodium chloride, 50 mM sodium phosphate, and 5 mM EDTA (pH 7.4)], 1% SDS, and salmon sperm DNA (100 μg/mL) with [α-^{32}P]dCTP-labeled cDNA probe. The probe used was a 0.42 kb full-length cDNA fragment of GSKIP. The blots were rinsed twice in 2× SSC and 0.1% SDS at room temperature for 10 min and washed twice in 0.1× SSC and 0.1% SDS at 50 °C for 20 min. The X-ray film was exposed overnight at −70 °C.

Protein. To generate various His-tagged GSKIP fusion proteins, GSKIP cDNA encoding GSKIP was introduced into the pET-32a (Novagen) vector. DNA fragments encoding GSKIP were amplified by PCR and inserted into the *Bam*HI and *Xho*I sites. To purify the His-tagged GSKIP, His-tagged $GSKIP_{1-108}$, His-tagged $GSKIP_{109-139}$, His-tagged GSKIP-(L130P) fusion proteins, 0.3 L of *Escherichia coli* BL21-(DE3) cells was grown to mid-log phase. The solution was induced at 37 °C using isopropyl 1-thio-β-D-galactopyranoside (IPTG), lysed by sonication in buffer A (20 mM Tris-HCl, 0.8% NaCl, and 0.1% lysozyme, supplemented with protease inhibitors), and purified by chromatography on Ni-charged agarose. After being washed, the His-tagged GSKIP fusion proteins were eluted with buffer containing 500 mM imidazole. Site-directed mutagenesis was performed on plasmids pET32a-GSKIP and pET32a-$GSKIP_{109-139}$ to create L130P, S109A, T113A, S115A, S109A/T113A, and S109A/T113A/S115A mutants. All mutants were sequenced to confirm that only the intended point mutation was introduced. All proteins used in this study were purified by the same method, and they were quantified with a Braford assay (Bio-Rad) using BSA as a standard.

GST Pulldown Assay. E. coli BL21(DE3) (pGEX-4T1-GSK3β, pGEX-4T1 vector) was cultured in 3 mL of LB medium at 37 °C to mid-log phase. IPTG was then added to a final concentration of 1 mM to induce the expression of the GST fusion protein. After being cultured for 3 h, cells were pelleted by centrifugation and suspended in 100 μL of a lysis buffer, B-Per (Pierce), containing 10 μL of leupeptin, aprotinin, and 4-(2-aminoethyl)benzenesulfonyl fluoride. The suspension was centrifuged again at 10 000 rpm for 5 min

at 4 °C using a T15A22 rotor in a Hitachi CF R15 centrifuge. Glutathione−Sepharose 4B beads (20 μL) (Amersham Pharmacia Biotech) were then added to the supernatant, and the mixture was incubated with shaking for 1 h at 4 °C. The beads were washed three times with NETN buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40]. After being washed, the beads were added to the lysate (300 μL) prepared from *E. coli* containing the various His-tagged GSKIP fragments. The reaction mixture was incubated on ice for 1 h to allow the binding of the GST fusion proteins, which included GST−GSK3β, His-tagged GSKIP(full-length), His-tagged GSKIP$_{1-108}$, His-tagged GSKIP$_{109-139}$, or His-tagged GSKIP(L130P) (*31*). The beads were subsequently washed with NETN buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 1% Tween 20]. An equal volume of 2× electrophoresis sample buffer was then added to the mixture, and proteins were extracted from the beads by heating them at 95 °C for 5 min. Proteins were finally analyzed by SDS−polyacrylamide gel electrophoresis, transferred to PVDF, and incubated for 1 h in blocking buffer (5% nonfat milk in PBS and 0.1% Tween 20). His or GST polyclonal antibodies were used as the primary antibodies to detect the appropriate proteins by incubation first in blocking buffer for 1 h at room temperature, and this was followed by incubation with the second HRP-conjugated anti-rabbit antibody for an additional 1 h.

Co-Immunoprecipitation. HEK293 cells transfected with pcDNA-vector, pcDNA-GSKIP, pcDNA-GSKIP(L130P), or pCMV-GSK3β 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. The lysate was then centrifuged with a microcentrifuge at 10000*g* for 20 min. Anti-Flag (Sigma) antibody was added to the supernatant and the mixture 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 finally collected by centrifugation and washed three times with radioimmune precipitation assay buffer. Proteins binding to the beads were eluted by adding 20 L of 2× electrophoresis sample buffer and analyzed by immunoblotting with an anti-HA antibody (Roche). Endogenous GSK3 immunoprecipitation was performed using an antibody raised against pan GSK3 (Santa Cruz).

In Vitro Kinase Assay. The kinase reaction was carried out as described previously (*32*, *33*). Briefly, the GSKIP variant protein was purified and incubated with GSK3β (25 units, NEB) in kinase buffer [1 mM Na_3VO_4, 1 mM dithiothreitol, 2 mM EGTA, 25 mM Tris (pH 7.2), 10 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM PMSF, 10% glycerol, and 10 Ci of [γ-^{32}P]ATP (Amersham), 3000 Ci/mM]. The assays were carried out for 15 min at 30 °C. For the GSK3β inhibiting assays, GSK3β was incubated with various concentrations of GSKIP for 15 min, and each substrate (including GST−Axin$_{275-510}$ from Upstate and GST−β-catenin, His-Tau, and glycogen synthase from Sigma) was added to the reaction mixture. The phosphorylation of β-catenin used 100 units of GSK3β (from NEB). The following concentrations of substrates were added to the reaction mixture: 25 μg/mL Axin, 0.125 mg/mL β-catenin, 50 μg/mL Tau, and 50 μg/mL glycogen synthase. The reaction was stopped by adding 2× sample buffer and heating at 95 °C for 5 min; this was followed by SDS−PAGE, and results were detected via autoradiography.

Cell Culture, Transfections, and Indirect Immunofluorescence. HeLa cells were grown at 37 °C in DMEM supplemented with 10% FCS and penicillin with streptomycin (100 IU/mL). For the transient transfection studies, HeLa cells were seeded onto glass coverslips at a density of 0.7×10^5 cells per 12-well plate. DNA (1 μg) was transfected into the HeLa cells using TransFast transfection reagent (Promega). After 24 h, the cells were fixed in cold methanol for 20 min and immunostained as described previously (*34*). The fixed cells were probed with anti-HA (Roach) polyclonal antibody, and the secondary antibodies were FITC-conjugated goat anti-rat antibodies (1:250, Santa Cruz). The fixed cells were also probed with the anti-β-catenin polyclonal antibody, and the secondary antibody was a rhodamine-conjugated goat anti-rabbit antibody (1:250, Santa Cruz). DNA was stained with DAPI (Roche) (*34*, *35*). An Olympus Fluoviews confocal microscope, based on an Olympus IX-70 inverted microscope, was used for microscopy.

Western Blot Analysis. For Western blot analysis, the HEK293 cell line was maintained in DMEM supplemented with 10% FBS. Cells were harvested 24 h after transfection and washed once in TBS. After that, the cells were resuspended in cell lysate 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)]. Samples were left for 30 min on ice and then centrifuged at 14 000 rpm for 5 min at 4 °C. The supernatant was then placed into a fresh centrifuge tube, protein sample buffer added, and the sample heated to 95 °C for 5 min; this was followed by analysis by 12% SDS−PAGE as previously described (*36*). 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). β-Catenin, 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.

Luciferase Reporter Assays. The β-catenin mutant (T41A/S45A) with mutations at positions 41 and 45 of β-catenin was made as previously described (*37*, *38*). Luciferase reporter plasmids were created by introducing four copies of the TCF4 DNA binding motif (CTTTCATC) from the cyclin D1 promoter into the pGL2B luciferase reporter plasmid (Promega). The HEK293 cell line was maintained in DMEM supplemented with 10% FBS. Each GSKIP construct was cotransfected with the pGL2B-TCF4 luciferase reporter plasmid. DNA transfections were performed using electroporation (Gene pulser II, Bio-Rad). The luciferase analysis was performed with Lucy 1 (Anthos) according to the manufacturer's protocol. Luciferase readout was always obtained from triplicate transfections and averaged. Luciferase activity was normalized against Renilla luciferase (Promega) as an internal control.

Affinity Sensor Analysis (39). The binding affinity of GSK3β and GSKIP or GSKIPtide was determined using an ANT 100 affinity sensor (ABgene). GSKIP or GSKIPtide

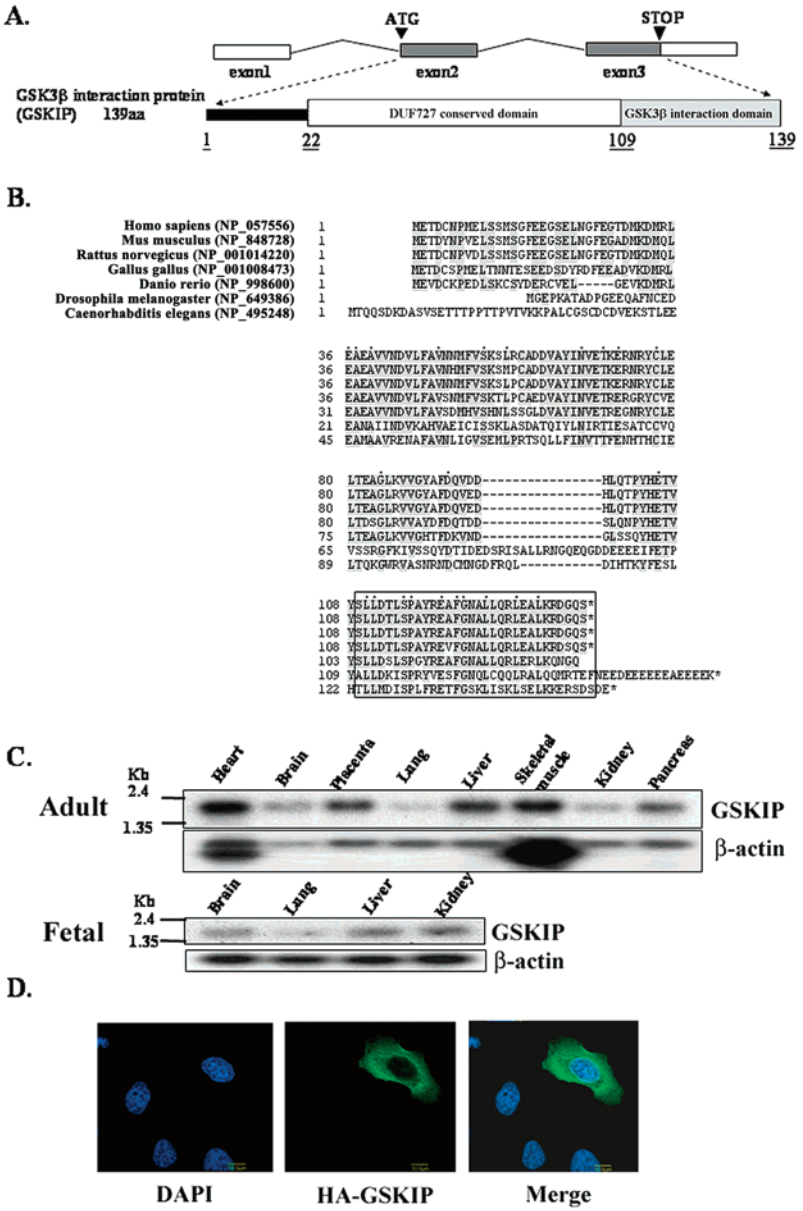

FIGURE 1: Protein sequence alignment of GSKIP and its subcellular localization. (A) Schematic representation of GSKIP and its domains. The genomic organization of GSKIP is shown with the three exons. (B) Protein sequence alignment of seven species. Note that C-terminal amino acids 109−139 of GSKIP ($GSKIP_{109-139}$) contain a highly conserved region across all seven species. (C) Northern blotting analysis of GSKIP expression in various human tissues. The membrane contained ~2 μg of poly(A^+) mRNA from each tissue. Hybridization was conducted using an α-^{32}P-labeled cDNA probe for full-length GSKIP with human β-actin as a control. (D) Localization of GSKIP.

was immobilized covalently via a primary amine group on the AM25 sensor chip (ABgene). Initially, the chip was activated with 2.5% glutadialdehyde in binding buffer [50 mM HEPES (pH 7.4) and 100 mM NaCl], and then 1 mM GSKIP or GSKIPtide was immobilized on the chip; the chip was next blocked using 1 M glycine. GSK3β at various concentrations in binding buffer was added to the chip and allowed to bind to the immobilized GSKIP or GSKIPtide. The affinity constant (K_d) was calculated from the real-time binding data using the provided AE software, version 1.0 (ABgene).

RESULTS

Molecular Cloning of GSKIP. To identify possible proteins involved in GSK3β binding, a human testis cDNA library (Clontech) was screened using the yeast two-hybrid system. One of the detected proteins was designated GSKIP (GSK3β interaction protein). The GSKIP cDNA sequence contains an open reading frame of 420 bp encoding a polypeptide of 139 amino acids with a predicted molecular mass of 15 648 Da (pI = 4.36) (Figure 1A). The GSKIP gene is located at chromosome 14q32.2 and organized into three exons (Figure 1A). GSKIP contains a novel domain of uncharacterized function (DUF727) that shows conservation. This conserved domain is retained from worm to human (Figure 1B). Sequence comparisons using GSKIP revealed that similarity is shown mainly in mouse, rat, chicken, and zebrafish, where there is >75% identity, whereas fly and worm are only 31 and 37% identical, respectively (Figure 1B). Interestingly, the C-terminal (amino acids 109−139) 31-amino acid region of GSKIP ($GSKIP_{109-139}$) is highly conserved in all species (Figure 1B, boxed region). By Northern blot analysis, a 2.1

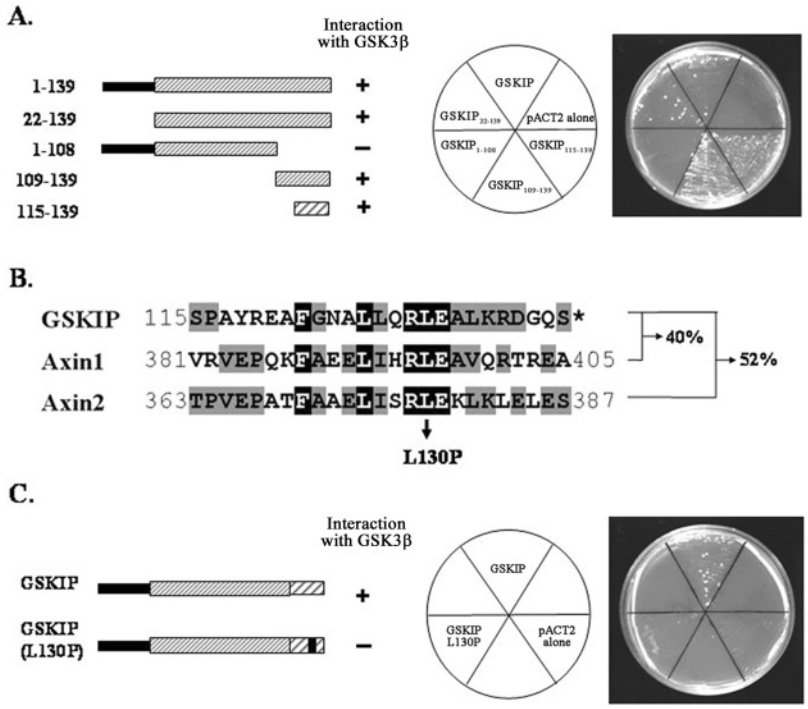

FIGURE 2: Twenty-five-amino acid sequence of GSKIP that is highly similar to the GID of Axin. (A) Serial deletion mutants of GSKIP indicating interaction with GSK3β: +, strong interaction; −, no interaction. (B) Amino acid sequence of $GSKIP_{115-139}$, which similar to the highly conserved region of $Axin1_{381-405}$ and $Axin2_{363-387}$. Amino acid similarities between GSKIP and Axin proteins are highlighted in gray. Amino acid identities are highlighted in black. (C) GSKIP(L130P) mutation that prevents association of GSK3β with GSKIP. Growth indicates a positive interaction.

kb transcript was found in almost all adult tissues and fetal tissues examined, and there is a relatively high expression level in the heart, placenta, liver, and skeletal muscle (Figure 1C). To identify the subcellular localization of GSKIP, HeLa cells expressing the HA−GSKIP fusion protein were examined by fluorescence microscopy. The data showed that most GSKIP is localized to the cytoplasm (Figure 1D).

A 25-Amino Acid Residue Sequence of GSKIP Is Highly Similar to the GID of Axin. To map the interaction region between GSKIP and GSK3β, we performed a yeast two-hybrid assay to clarify whether the interaction was affected by the DUF727 conserved domain. The regions of GSKIP that were investigated are shown in Figure 2A. $GSKIP_{115-139}$ is the smallest fragment that is sufficient for GSK3β binding (Figure 2A). The data provide evidence that C-terminal region of GSKIP is able to interact with GSK3β, a region which is highly conserved in all species (Figure 1B). Since this 25-amino acid residue sequence of GSKIP can bind GSK3β, we thus suggest that this small fragment may act as a short GSK3β binding region for GSK3β substrates. Previous reports have shown that $GID_{381-405}$ (a 25-amino acid region derived from Axin) is critical to association with GSK3β (*24*). Interestingly, $GSKIP_{115-139}$ is highly similar to $GID_{381-405}$ of Axin (Figure 2B). $GSKIP_{115-139}$ is 32% identical (40% similar) to $Axin1_{381-405}$ and is 36% identical (52% similar) to $Axin2_{363-387}$. Recent results have shown that an Axin mutation in which leucine 396 is changed to proline, when introduced into the putative hydrophobic interface of the coiled-coil domain, blocks interaction of Axin with GSK3β (*26*, *40*). Thus, we also mutated the corresponding leucine 130 to proline in GSKIP. The yeast two-hybrid data show that the GSKIP(L130P) mutant also does not interact with GSK3β (Figure 2C). This suggests that GSKIP possesses a 25-amino acid residue function similar to that of $GID_{381-405}$ of Axin that allows association with GSK3β.

GSKIP Interacts with GSK3β in Vitro and in Vivo. To further confirm this protein−protein interaction, GSKIP and GSK3β were overexpressed to allow an in vitro binding assay to be carried out. Purified His-tagged GSKIP, His-tagged $GSKIP_{1-108}$, His-tagged $GSKIP_{109-139}$, and His-tagged GSKIP-(L130P) fusion proteins were analyzed by SDS−PAGE (Figure 3A). The results of the in vitro pulldown assay showed that GSKIP and $GSKIP_{109-139}$ bind to the GST−GSK3 protein, but not $GSKIP_{1-108}$ or GSKIP(L130P) (Figure 3B). The interaction between GSKIP and GSK3β was also established using an in vivo co-immunoprecipitating assay. HA empty vector, HA−GSKIP, or HA−GSKIP(L130P) was cotransfected into HEK293 cells with Flag−GSK3β. As shown in Figure 3C, HA−GSKIP was able to co-immunoprecipitate with Flag−GSK3β. Moreover, the immunoprecipitation with HA−GSKIP and an antibody raised against pan GSK3 yields the same results (Figure 3D). It is noted that GSK3α also binds to GSKIP. However, HA−GSKIP-(L130P) failed to co-immunoprecipitate with GSK3β (Figure 3C,D). In addition, the affinity of binding between GSKIP and GSK3β was also demonstrated using the Biacore system (data not shown), which determined the binding affinity between GSKIPtide and GSK3β to be strong at 2.7 μM, which is very close to an affinity of 3.2 μM in the Axin GID (*41*). Together, there is agreement between the in vivo results, the yeast two-hybrid assay (Figure 2C), the in vitro biochemical analyses, and the Biacore affinity assay, which confirms that GSKIP and GSK3β are able to interact with each other.

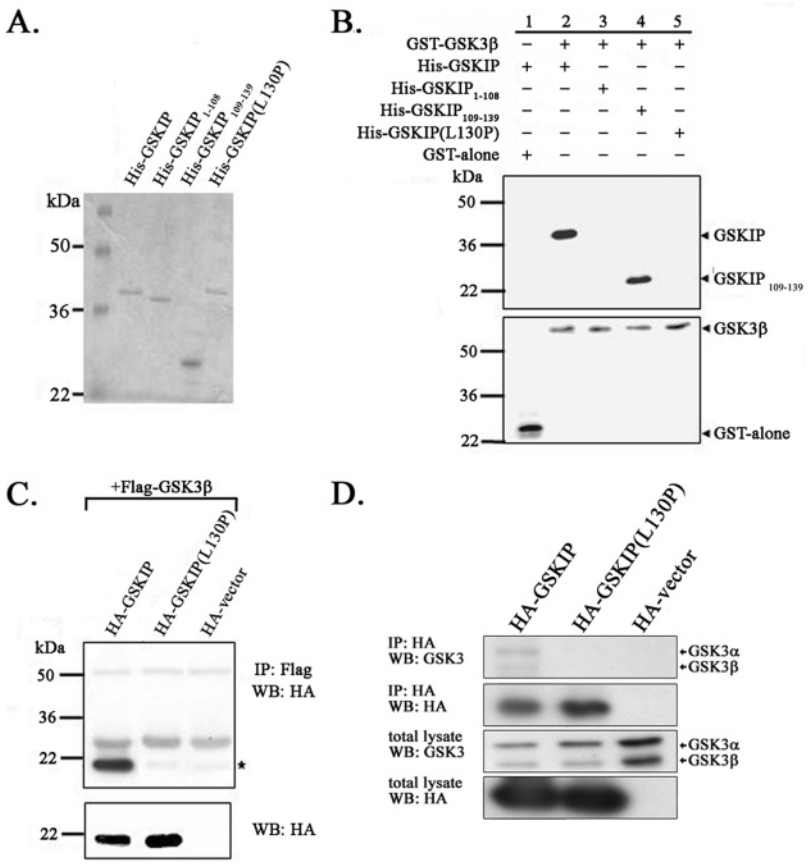

FIGURE 3: GSKIP interacts with GSK3 in vivo and in vitro. (A) Coomassie blue staining of GSKIP constructs. (B) GST pulldown analysis of GSKIP with GSK3β. The two fusion proteins were tested for coelution from glutathione−Sepharose 4B beads, which would indicate interaction between GSKIP(full-length) or $GSKIP_{109-139}$ and GSK3β. (C) Co-immunoprecipitation of GSKIP with GSK3β. HEK293 cells were cotransfected with pCMV-Flag-GSK3β and/or pcDNA-GSKIP or pcDNA-GSKIP(L130P) or pcDNA vector. Immunoprecipitation (IP) was performed with an anti-Flag antibody. Western blotting (WB) was performed using an anti-HA antibody. A star indicates the GSKIP signal. (D) Endogenous GSK3 interacts with GSKIP. HEK293 cells were transfected pcDNA-GSKIP, pcDNA-GSKIP(L130P), or pcDNA vector. Immunoprecipitation (IP) was performed with an anti-HA antibody. Western blotting (WB) was performed using an anti-GSK3 or anti-HA antibody.

GSK3β Phosphorylates GSKIP. To further examine whether GSKIP is a substrate for GSK3β, we performed an in vitro kinase assay. Our results showed that GSK3β can phosphorylate native GSKIP (Figure 4A, lane 1). To determine which regions of GSKIP were phosphorylated by GSK3β, several His-tagged fusion proteins were tested. The results indicated that the C-terminus of GSKIP ($GSKIP_{109-139}$), but not the N-terminus of GSKIP ($GSKIP_{1-108}$), was highly phosphorylated by GSK3β (Figure 4A, lanes 2 and 3). As described above, fragments of amino acids 109−139 or 115−139 of GSKIP are sufficient for GSK3β binding (Figure 2C). GSKIP(L130P) failed to be phosphorylated by GSK3β, indicating that GSKIP has to interact with GSK3β for phosphorylation (Figure 4A, lane 4). To further confirm that the GSK3β phosphorylation consensus sequences are within the C-terminal region [putative GSK3β phosphorylation sites, Ser/Thr-X-X-X-Ser/Thr and Ser/Thr-Pro (Figure 4B)], we performed site-directed mutagenesis on GSKIP. Single mutation of any of the putative GSK3β target sites (Ser109, Thr113, or Ser115) to alanine failed to yield any significant changes in the level of phosphorylation (Figure 4C). However, the double mutation (S109A/T113A) or the triple mutation (S109A/T113A/S115A) of GSKIP showed levels of phosphylation significantly reduced to 45% in fragment 109−139 and 30% in full-length GSKIP (Figure 4C). These results indicate that Ser109 and Thr113 are major GSK3β phosphorylation targets. It should be noted that the C-terminal (amino acids 109−139) 31-amino acid region of GSKIP ($GSKIP_{109-139}$) is highly conserved in all species (Figure 1B, boxed region). Our data indicate that the C-terminal (amino acids 109−139) region of GSKIP contains two distinct regions: phosphorylated sites (amino acids 109−115) and the GSK3β interaction region (amino acids 115−139) which is highly similar to the $GID_{381-405}$ of Axin (Figure 4B; see also the section below). It is still unclear why the single mutations of Ser109 and Thr113 did not affect phosphorylation by GSK3β. One possible explanation is that a "priming" phosphorylation is not a prerequisite for GSK3β to phosphorylate GSKIP in vitro. It should also be noted that the invertebrates do not contain all three phosphorylation sites. This is particularly surprising since the one conserved site in *Drosophila* is the one that seems not be essential in vertebrates (Ser115, Figure 1B).

GSKIP Competes with and Inhibits GSK3β Activity. Klein's laboratory has shown that two constructs encoding 110 amino acids ($GID_{320-429}$) and 25 amino acids ($GID_{381-405}$) derived from the GSK3β interaction domain (GID) of Axin could selectively inhibit GSK3β phosphorylation of non-primed substrates (*26*). Our data indicate that $GSKIP_{115-139}$ functions in a manner similar to that of $GID_{381-405}$ of Axin and is involved in association with GSK3β. An in vitro kinase assay was performed to test whether GSKIP competed

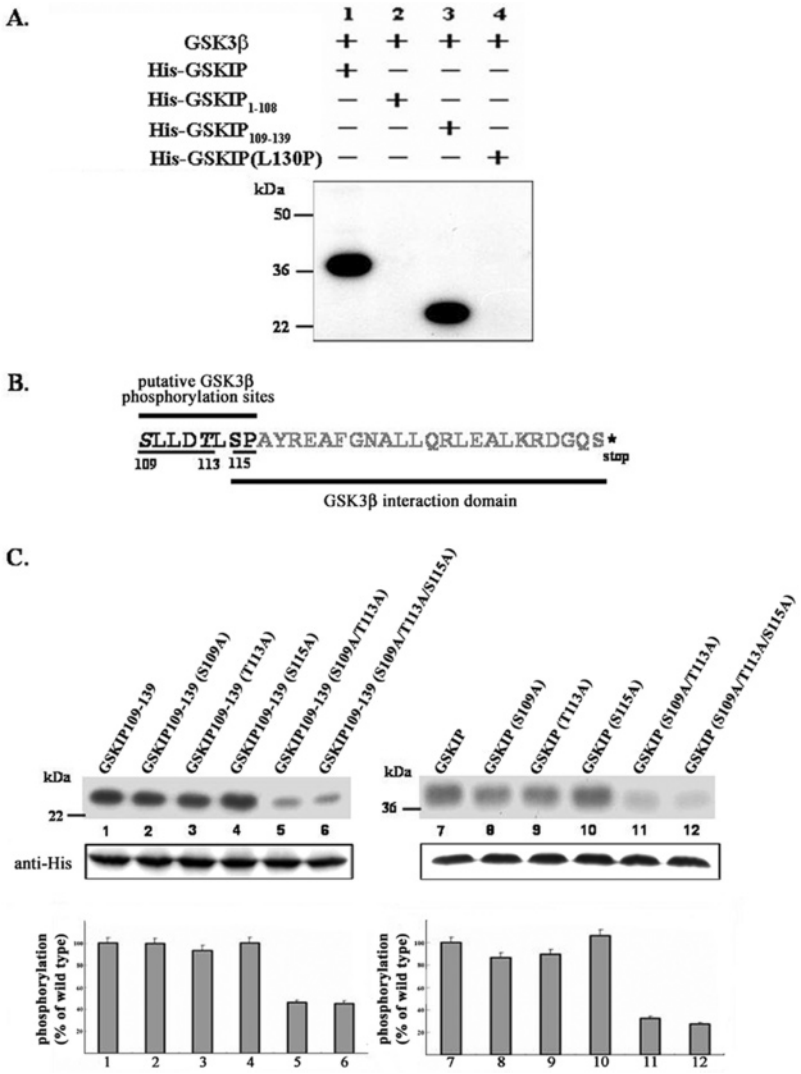

FIGURE 4: GSK3β phosphorylates GSKIP at S109 and T113. (A) The kinase assay was performed using purified GSKIP, GSKIP$_{1-108}$, GSKIP$_{109-139}$, GSKIP(L130P), and GSK3β. (B) Schematic diagram of three putative phosphorylation sites (Ser109, Thr113, and Ser115) and the GSK3β interaction domain (residues 115−139) of GSKIP$_{109-139}$. (C) To perform kinase assays, we tested the wild type (lane 1), S109A (lane 2), T113A (lane 3), S115A (lane 4), S109A/T113A (lane 5), and S109A/T113A/S115A (lane 6) for GSKIP$_{109-139}$ phosphorylation by GSK3β. GSKIP(full-length), wild type (lane 7), S109A (lane 8), T113A (lane 9), S115A (lane 10), S109A/T113A (lane 11), and S109A/T113A/S115A (lane 12) also underwent phosphorylation by GSK3β. Bars below the lanes show equal amounts of GSKIP$_{109-139}$ and GSKIP-(full-length) were detected by Western blotting as a control. Phosphorylation quantified by BIO-PROFIL Bio-1D. Data are presented as means ± the standard error from three independent experiments, each performed in duplicate.

with Axin phosphorylation by GSK3β. His-tagged GSKIP-(full-length) fusion protein and GST−Axin$_{275-510}$ protein were added to the in vitro kinase assay. Our data showed that GSKIP could prevent GSK3β-catalyzed phosphorylation of GST−Axin$_{275-510}$ protein, in a manner similar to that of GID$_{320-429}$ of Axin (Figure 5A). In addition, the His-tagged vector protein could not prevent the GSK3β-catalyzed phosphorylation of GST−Axin$_{275-510}$ protein (Figure 5A, left panel, lane 5). GSK3β-catalyzed phosphorylation of β-catenin could be promoted by Axin (*42*), and our data also showed that Axin-dependent phosphorylation of β-catenin could also be blocked by GSKIP (Figure 5B). Moreover, we were able to demonstrate the ability of GSKIP to inhibit GSK3β in vitro using a range of substrates, including Tau and glycogen synthase (GS). Tau is a microtubule-binding protein, and it is identified as a GSK3 substrate involved in Alzheimer's disease. GS is involved in glycogen synthesis, and it is well-known for its ability to be phosphorylated by GSK3β. Our data show that GSKIP could inhibit GSK3β phosphorylation of Tau, but not GS (Figure 5C,D). Equally, the mutant GSKIP(L130P) did not compete with or inhibit phosphorylation of these substrates (data not shown). These results suggest that the GSKIP function is to compete with and/or inhibit GSK3β kinase activity through direct binding.

Synthesized GSKIPtide Could Function as an Inhibitor of GSK3β. Abnormal GSK3β activity may be associated with and result in a range of human diseases. A well-established inhibitor of GSK3β is lithium, which is fairly specific for GSK3β (*43*); however, lithium also affects other enzymes. Large doses of lithium are required to inhibit GSK3β activity in cell culture (*43*, *44*), and therefore, the search for better GSK3β inhibitors or binding proteins has become important. Recently, it has been reported that a peptide derived from Axin GID$_{381-405}$ could as an inhibitor of GSK3 (*26*). Our results showed that C-terminal region of GSKIP is highly similar to the GID$_{381-405}$ of Axin. Therefore, we first tested whether the C-terminus (amino acids 109−139) of GSKIP inhibits GSK3 activity. As expected, the data showed that

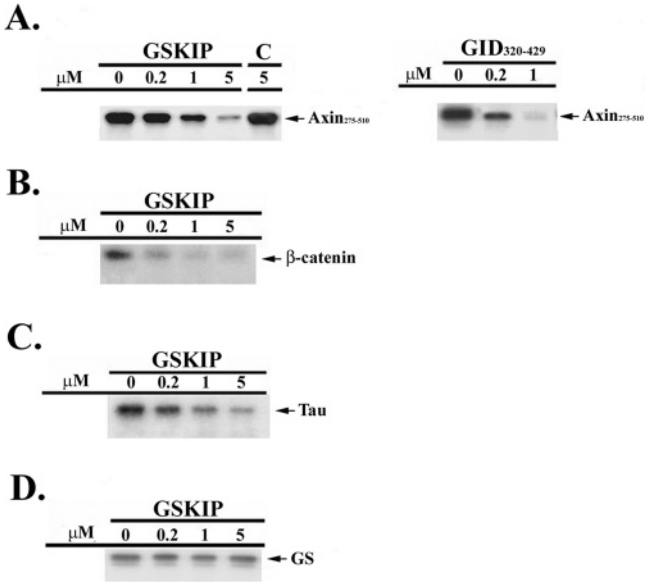

FIGURE 5: GSKIP directly inhibits GSK3 activity. Four reactions were analyzed in the presence of assay mixtures containing recombinant GST−$Axin_{275-510}$ protein, GST−β-catenin protein, His-tagged Tau, or glycogen synthase (GS) and GSK3β: (A) GST−$Axin_{275-510}$, (B) GST−β-catenin (in the presence of $Axin_{275-510}$), (C) His-tagged Tau, and (D) GS. All reaction mixtures contained GSKIP at various doses (0, 0.2, 1, and 5 μM). The arrow indicates phosphorylated GST−Axin protein (amino acids 275−510), His-tagged Tau, and GST−β-catenin protein. The arrowhead indicates phosphorylated GSKIP. In panel A, lane C indicates the His-tagged vector protein, which acts as a negative control. $GID_{320-429}$ (right panel) contains various doses of the peptide (0, 0.2, and 1 μM) and acts as a positive control.

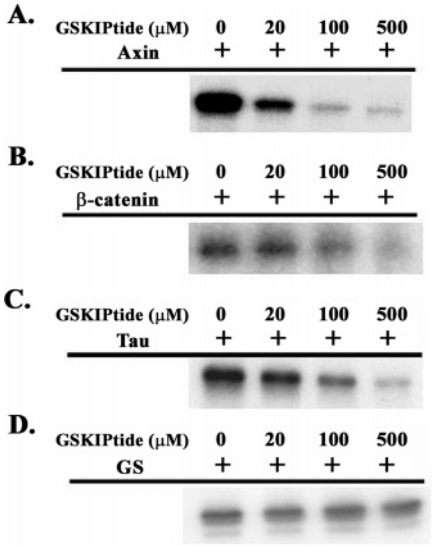

FIGURE 6: Synthesized GSKIPtide acts as an inhibitor. Four reactions were analyzed in the presence of assay mixtures containing recombinant GST−$Axin_{275-510}$ protein, GST−β-catenin protein, His-tagged Tau, or glycogen synthase (GS) and GSK3β: (A) GST−$Axin_{275-510}$, (B) GST−β-catenin (in the presence of $Axin_{275-510}$), (C) His-tagged Tau, and (D) GS. All reaction mixtures contained various doses of GSKIPtide (0, 20, 100, and 500 μM).

the C-terminus of GSKIP also prevented GSK3β-catalyzed phosphorylation of Axin (data not shown). We then synthesized a peptide (SPAYREAFGNALLQRLEALKRDGQS) derived from $GSKIP_{115-139}$, which was designated GSKIPtide, to test the peptide's ability to inhibit GSK3β-catalyzed phosphorylation. These data showed that GSKIPtide also blocks phosphorylation by GSK3β of the tested nonprimed substrates, Axin, β-catenin, and Tau, but not primed GS (compare panels A−C of Figure 6 to panel D). Therefore, we concluded that a 25-residue peptide from the C-terminus of GSKIP, GSKIPtide, acts as an inhibitor.

GSKIP Functions as a Negative Regulator of GSK3β in the Wnt Pathway. GSK3β is a key regulatory kinase in the Wnt signaling pathway, where it phosphorylates β-catenin and marks β-catenin for proteasomal degradation (*42*). Interaction of GSK3β with Axin in the complex facilitates efficient phosphorylation of β-catenin by GSK3β (*45*). As shown in Figure 2B, we found that GSKIP possesses a 25-amino acid residue sequence similar to $GID_{381-405}$ of Axin. Klein's laboratory has observed that overexpression of $GID_{320-429}$ or $GID_{381-405}$ causes an accumulation of β-catenin (*26*). We therefore tested whether GSKIP caused the accumulation of β-catenin in HeLa cells. GSKIP-induced β-catenin accumulation was evident in both the cytoplasm and the nucleus, as visualized by immunofluorescence detection of β-catenin in HeLa cells (Figure 7A). Further results showed that while GSKIP caused β-catenin accumulation, the mutant GSKIP(L130P) or vector alone did not cause β-catenin accumulation (Figure 7A). By Western blot analysis, expression of wild-type GSKIP, but not the mutant GSKIP(L130P), caused β-catenin accumulation (Figure 7B, left panel). Accumulation of β-catenin allows entry into the nucleus, where interaction of β-catenin with Tcf/Lef family transcription factors stimulates expression of cellular genes whose promoters contain Tcf/Lef binding sites (*4*, *46*, *47*). To determine whether antagonism of the GSK3β function by GSKIP led to an increase in nuclear β-catenin activity, transient expression assays were performed in HEK239 cells using a luciferase reporter assay. These results showed that wild-type GSKIP could affect Wnt-dependent transcription using a TCF/Lef luciferase reporter but the mutant GSKIP(L130P) did not exhibit a significant difference between the construct plasmids and the wild type (Figure 7B), while typical β-catenin double mutant T41A/S45A, which stabilizes β-catenin, exhibited significantly higher activity as expected (Figure 7B, right panel). In addition, we also showed that GSKIP could activate reporter assays in a dose-dependent manner (Figure 7C). Altogether, these results suggest that GSKIP may be involved in the Wnt signaling pathway.

DISCUSSION

The wide role of GSK3β has suggested that the enzyme is involved in multiple cellular processes, including glycogen metabolism, gene expression, proliferation, and development (*1*−*4*, *48*−*51*). Here, we report the cloning and characterization of another naturally occurring GSK3β interaction protein (GSKIP), the C-terminus of which contains a domain similar to the GID of Axin. We also demonstrate that the function of GSKIP is similar to that of the prominent GSK3β binding protein, FRAT/GBP. Moreover, similar to Axin $GID_{381-405}$ and FRATtide, a synthesized GSKIPtide is also shown to compete with and/or block GSK3β-catalyzed phosphorylation of Axin and β-catenin involved in the Wnt signaling pathway.

The $GSKIP_{115-139}$ Region of GSKIP and $GID_{381-405}$ of Axin Are Similar. Axin is a scaffold protein that binds multiple components of the canonical Wnt pathway. It is also shown

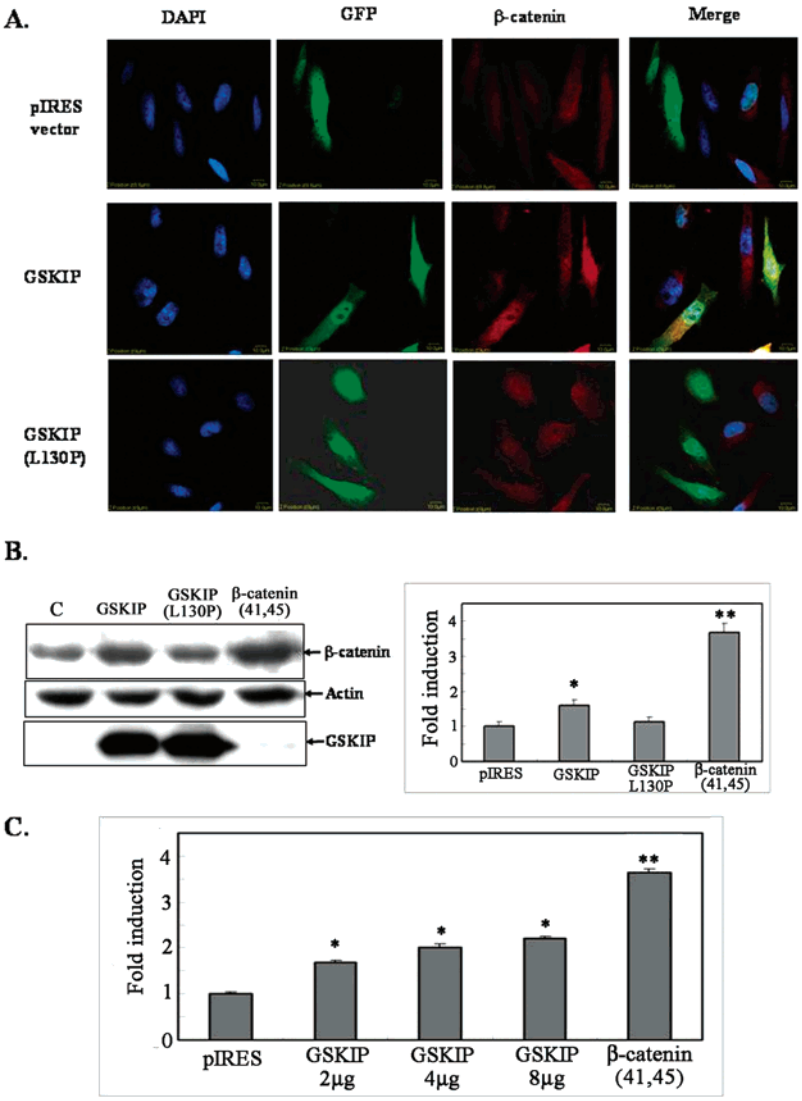

FIGURE 7: GSKIP causes β-catenin accumulation in the cytoplasm and nucleus and activates the reporter systems. (A) GSKIP induces accumulation of β-catenin in the cytoplasm and nucleus as visualized by immunofluorescence. HeLa cells were cotransfected with GSKIP, GSKIP(L130P), or pIRES vector, together with pEGFP. GSKIP expression is indicated in the transfected cells by green. β-Catenin is stained with rhodamine-conjugated secondary antibody and is colored red. Nuclei are stained with DAPI (blue). (B) HEK293 cells were transfected with 2 μg of wild-type β-catenin with pIRES-GSKIP, pIRES-GSKIP(L130P), or pIRES vector and compared with a positive control, β-catenin (*41*, *45*). Accumulation of β-catenin in the presence of GSKIP in HEK293 cells was assessed. β-Catenin was detected by Western blotting (left panel). Fold induction indicates transcriptional activity compared with pIRES vector control plasmid (right panel). (C) HEK293 cells were transfected with increasing concentrations of GSKIP as indicated. Each value represents the mean $\pm$ the standard deviation of three separate experiments. Statistically significant differences as determined by a Student's *t* test: $p < 0.005$ (one asterisk) and $p < 0.0005$ (two asterisks) vs control.

that Axin acts as a negative regulator of the Wnt signaling pathway by interacting with several proteins, including GSK3β, APC, β-catenin, and Dvl, and by stimulating the degradation of β-catenin (*52*). A crystal structure of GSK3β bound to the 25-amino acid residue peptide from Axin $GID_{381-405}$ has been published recently that confirms the GSK3β interaction site (*53*). Axin residues Phe388, Leu392, Leu396, and Val399 form a hydrophobic helical ridge that packs into a hydrophobic groove formed between helix $GSK3\beta_{262-273}$ and extended loop $GSK3\beta_{285-299}$. In this paper, we show that the two sequences, $GSKIP_{115-139}$ and $GID_{381-405}$ (Figure 2B), are similar and that the residues in GSKIP corresponding approximately to Phe388, Leu392, Leu396, and Val399 of Axin are Phe122, Leu126, Leu130, and Leu133, respectively. Indeed, our data also show that the mutant GSKIP(L130P) (corresponding to Axin Leu396) results in a loss of interaction with GSK3β (Figures 2C and 3). This is not the case with FRAT/GBP, which lacks such similarity in sequence with Axin and only through an overlapping binding region prevents the GSK3β-catalyzed phosphorylation of $Axin_{275-510}$ (*19*, *25*, *54*). It seems that GSKIP or GSKIPtide is shown to prevent GSK3β-catalyzed phosphorylation of $Axin_{275-510}$ possibly via binding site overlap or sequence similarity. Thus, it appears that there are at least two modes whereby GSKIP selectively inhibits GSK3β phosphorylation. These results thus support the hypothesis that another naturally occurring GSK3β binding protein, GSKIP or GSKIPtide, is able to block the GSK3β-catalyzed phosphorylation of Axin not only via sequence homology but also via binding site overlap.

The Function of GSKIP Is Similar to That of FRAT/GBP, Which Acts as a Negative Regulator. In previous studies,

among a large group of GSK3β interaction proteins, it has been shown that only two proteins, FRAT/GBP and p24, inhibit GSK3β (*22*, *55*). p24 has been reported to act as a GSK3β binding protein, but it is not a good GSK3β substrate. Despite the fact that p24 can affect GSK3β activity, how it regulates GSK3β activity is not clear. FRAT/GBP is a prominent GSK3β binding protein that can also inhibit the ability of GSK3β to phosphorylate substrates (*22*, *25*). It should be noted that the ability of GSKIP to inhibit Axin, Tau, and β-catenin phosphorylation but not phosphorylation of GS is the same as that for FRAT/GBP (Figure 5). It appears that these proteins do not possess a priming phosphorylation site, while in GS, priming phosphorylation is present (*44*, *56*). Further, two peptides derived from FRAT/GBP and GID can bind GSK3β and prevent substrate phosphorylation by GSK3β (*26*). In this study, we have found a novel GSK3β binding protein, GSKIP, which seems to act through a GID-like domain act as an inhibitor and regulate GSK3β activity. Indeed, GSK3 preferentially phosphorylates proteins and peptides at serine or threonine residues, and this is followed by another phosphoserine, frequently up to a total of four residues at the C-terminus of the GSK3 site (*4*, *16*, *25*, *26*, *57*). Interestingly, like Axin $GID_{381-405}$ and FRATtide, our protein or synthesized GSKIP peptide acts on the various proteins (Axin, β-catenin, and Tau) that are phosphorylated by GSK3, but GS is unaffected by GSKIPtide (Figure 6). We therefore conclude that the function of GSKIP is to act as a negative regulator of GSK3β and GSKIP is similar to FRAT/GBP.

GSKIP May Participate in the Wnt Signaling Pathway. It is well-known that Axin acts as a negative regulator in the Wnt signaling pathway by interacting with several proteins, including GSK3β, APC, β-catenin, and Dvl, and by stimulating the degradation of β-catenin. In mammalian cells, Wnt stimulation can activate Dvl, which with FRAT can disrupt Axin−GSK3β interaction. In addition, FRAT causes GSK3β to move away from Axin and β-catenin (*4*, *16*, *18*−*20*, *26*, *27*). In this report, we show overexpression of GSKIP causes β-catenin accumulation. Conversely, the mutant GSKIP-(L130P) or vector alone does not cause β-catenin accumulation (Figure 7). Our results also show that β-catenin binds to Tcf4/Lef transcription factors; furthermore, downstream responsive genes can be activated (Figure 7B,C). On the basis of this, we propose that GSKIP can compete with and displace Axin from GSK3β by binding to an overlapping site, which results in the breakup of the Axin−GSK3β−β-catenin complex. In the Wnt signaling pathway, we suggest that GSKIP may function like FRAT/GBP (despite the lack of sequence similarity between FRATtide and GSKIPtide) and that it may substitute for FRAT to participate in the GSK3β−Axin−β-catenin complex. Recent results have also shown that mice lacking all FRAT family members appear to be normal and display no obvious defects in β-catenin−TCF signaling. These studies show that FRAT is not an essential component of the canonical Wnt pathway in higher organisms, despite the strict requirement for FRAT/GBP in *Xenopus* maternal Wnt signaling (*58*, *59*) This observation re-opens the question of how GSK3 activity is controlled during vertebrate canonical Wnt signaling transduction in view of the apparent dispensability of FRAT. As seen in the model presented here, it is quite possible to see how there can be functional compensation whereby GSKIP can replace FRAT in the canonical Wnt pathway, since signaling would be unaffected by the loss of FRAT. This could also explain the conservation of this GSKIP protein throughout evolution rather than FRAT/GBP only in higher vertebrates (Figure 1B) (*59*).

CONCLUSIONS

In summary, we have identified a naturally occurring GSK3β binding protein, designated GSKIP (GSK3β interaction protein), whose C-terminal region possesses a 25-amino acid region similar to $GID_{381-405}$ of Axin. This region is required for GSK3β binding. The function of GSKIP is also similar to that of FRAT/GBP (despite the lack of sequence similarity between FRATtide and GSKIPtide), and our results indicate that GSKIP and GSKIPtide may act as an inhibitor of GSK3β and thus may also participate in the GSK3β−Axin−β-catenin complex as part of the Wnt signaling pathway. The discovery of GSKIP protein could also explain the conservation of this protein throughout evolution rather than FRAT/GBP only in higher vertebrates. Furthermore, to some extent, GSKIP and GSKIPtide as inhibitors of drug discovery of GSK3β need more exploration.

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