Glycogen Synthase Kinase 3β Interacts with and Phosphorylates the Spindle-associated Protein Astrin*

Received for publication, August 15, 2007, and in revised form, November 30, 2007 Published, JBC Papers in Press, November 30, 2007, DOI 10.1074/jbc.M706794200

Tai-Shan Cheng‡§, Yun-Ling Hsiao‡§, Ching-Chih Lin‡§, Chang-Tze Ricky Yu, Ching-Mei Hsu, Mau-Sun Chang**, Chu-I Lee††, Chi-Ying F. Huang‡§, Shen-Long Howng‡‡, and Yi-Ren Hong‡‡‡

From the ‡Graduate Institute of Biotechnology and §Graduate Institute of Medicine, Kaohsiung Medical University and ‡Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung 807, Taiwan, ‡Graduate Institute of Biomedicine and Biomedical Technology, National Chi Nan University, Nantou 545, Taiwan, ‡‡Department of Medical Research, Mackay Memorial Hospital, Taipei 104, Taiwan, ‡§Department of Medical Technology, Fooyin University, Kaohsiung 831, Taiwan, ‡¶Institute of Clinical Medicine, National Yang-Ming University, Taipei 112, Taiwan, and ‡¶¶Department of Neurosurgery, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan

Emerging evidence shows that glycogen synthase kinase 3β (GSK3β) is involved in mitotic division and that inhibiting of GSK3β kinase activity causes defects in spindle microtubule length and chromosome alignment. However, the purpose of GSK3β involvement in spindle microtubule assembly and accurate chromosome segregation remains obscure. Here, we report that GSK3β interacts with the spindle-associated protein Astrin both in vitro and in vivo. Additionally, Astrin acts as a substrate for GSK3β and is phosphorylated at Thr-111, Thr-937 ((S/T)P motif) and Ser-974/Thr-978 ((S/T)XXX(S/T)-p motif; p is a phosphorylatable residue). Inhibition of GSK3β impairs spindle and kinetochore accumulation of Astrin and spindle formation at mitosis, suggesting that Astrin association with the spindle microtubule and kinetochore may be dependent on phosphorylation by GSK3β. Conversely, depletion of Astrin by small interfering RNA has no detectable influence on the localization of GSK3β. Interestingly, in vitro assays demonstrated that Astrin enhances GSK3β-mediated phosphorylation of other substrates. Moreover, we showed that coexpression of Astrin and GSK3β differentially increases GSK3β-mediated Tau phosphorylation on an unprimed site. Collectively, these data indicate that GSK3β interacts with and phosphorylates the spindle-associated protein Astrin, resulting in targeting Astrin to the spindle microtubules and kinetochores. In turn, the GSK3β-Astrin complex may also facilitate further physiological and pathological phosphorylation.

Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase active in several signaling pathways, is involved in the regulation of cell fate, including Wnt and Hedgehog signal transduction, protein synthesis, glycogen metabolism, mitosis and apoptosis (1–4). GSK3 has two structurally similar isoforms in mammals, GSK3α and GSK3β, that are ubiquitously expressed and differ in their N- and C-terminal regions (2, 5). Earlier reports indicated that not only are the developmental profiles of GSK3α and GSK3β expression different but also the regulation and functions of these two proteins are not always identical (6–9). Factors known to influence the functions of GSK3β include the phosphorylation of GSK3β itself, the subcellular localization of GSK3β, the protein-protein interaction of GSK3β, and the phosphorylation state of GSK3β substrates (1, 3, 4, 10). Insulin-mediated inhibition of GSK3 was mediated through a phosphorylation-dependent mechanism, with the phosphorylation at position Ser-21 and Ser-9 in GSK3α and GSK3β, respectively (11).

GSK3β also has a preference for pre-phosphorylated substrates, recognizing the consensus sequence (S/T)XXX(S/T)-p. In this sequence the first S/T residue is the target for GSK3β phosphorylation, X is any amino acid, P denotes the phosphorylatable residue, and the S/T located at the C terminus is the phosphorylation priming site (12). On the other hand, a number of proteins, including Axin and Tau, are phosphorylated by GSK3β without pre-phosphorylation. Recombinant Tau is phosphorylated at Ser-396 by GSK3β directly at an (S/T)P motif (3, 13, 14). In fact, GSK3β phosphorylates many microtubule-associated proteins, including Tau, MAP1B, MAP2, APC, CRMP-2, and neurofilament, to regulate microtubule growth and stability and reduce their binding ability with microtubules (2, 3, 15, 16). Therefore, establishing and dynamic balancing of the microtubules depends on spatial-temporal regulation by GSK3β activity.

Microtubules (MTs) dynamics need to be controlled through the cell cycle, especially in spindle apparatus assembling for successfully segregating chromosomes. In mammals, GSK3β involvement has been reported in the polarized anchoring of MTs at the cell periphery (17). Furthermore, other recent studies found that GSK3β interacts with spindle apparatus during mitosis and accumulates at centrosomes and inhibiting GSK3β causes defects in extended astral microtubules and chromosome misalignment during mitosis (18, 19). Thus, identifying the interacting partners and candidate substrates for GSK3β in spindle microtubule assembly has great importance.
Astrin is a non-motor spindle-associated protein essential for cell cycle progression (20–22). This protein has a large coiled-coil domain at its C terminus and forms dimers or high order structures under physiological conditions (22). Interestingly, Astrin-depleted cells exhibit centrosome instability and malformation of the spindle, leading to mitosis arrest and subsequent apoptosis (20, 22, 23). A recent study also showed that in the absence of Astrin, kinetochore-microtubule attachments are impaired, leading to premature sister chromatid separation and centriole disengagement (24). These findings indicate that Astrin is important to spindle apparatus organization and chromosome segregation.

To explore the molecular targets for GSK3β that are involved in spindle microtubule assembly and accurate chromosome segregation, this investigation used a biochemical approach to search for proteins that specifically interact with GSK3β. This work presents a conceptual framework linking GSK3β and spindle-associated protein Astrin and shows that the association of Astrin with spindle MTs and kinetochore requires GSK3β kinase. In particular, the GSK3β-Astrin complex increases GSK3β-mediated phosphorylation on unprimed sites, suggesting that this molecular mechanism may be a recurring theme in spindle regulation.

**EXPERIMENTAL PROCEDURES**

All restriction enzymes were purchased from New England Biolabs. Fetal bovine serum, Dulbecco’s modified Eagle’s medium, penicillin, streptomycin, and LipofectamineTM were purchased from Invitrogen.

**Plasmid Constructions—** Astrin fragments were amplified from plasmid DNA by PCR, cloned into pAS2-1 (Clontech), and completely sequenced. Plasmids pET-Astrin, pET-Astrin aa 1–608, pET-Astrin aa 609–1193, aa pET-Astrin 1–478, and pET-Astrin aa 479–1193, which express His-tagged Astrin, His-tagged N terminus-Astrin (corresponding to amino acids 1–608), His-tagged C terminus-Astrin (corresponding to amino acids 609–1193), His-tagged Astrin aa 1–478, and His-tagged Astrin aa 479–1193 in Escherichia coli BL21(DE3), respectively, were constructed by inserting Astrin DNA fragments into the BamHI and XhoI sites, BamHI and EcoRI sites and EcoRI and XhoI sites of pET-32a (Novagen). Plasmid pcDNA-Astrin, pcDNA-Astrin aa 479–1193, and pcDNA-Astrin aa 609–1193 express hemagglutinin (HA)-tagged Astrin, HA-tagged Astrin aa 479–1193, and HA-tagged Astrin aa 609–1193, respectively. Plasmid GFP-Astrin, which expresses GFP-tagged Astrin protein, was constructed using the pEGFPC2 vector (Clontech) ligated at the restriction sites BglII and XhoI. Human GSK3β was fused to the GAL4 transcription activation domain of the yeast two-hybrid system (25–27). Briefly, the Astrin gene fragments were cloned in-frame with the GAL4 DNA binding domain in the pAS2-1 vector (MATCHMAKER Two-Hybrid System, Clontech). The human GSK3β was fused to the GAL4 transcription activation domain by subcloning into the pACT2 vector (MATCHMAKER Two-Hybrid System). Yeast YRG-2 (Stratagene) was co-transformed with the pAS2-1-Astrin plasmid DNA (GAL4 BD) and pACT2-GSK3β plasmid DNA (GAL4 AD). Positive clones were selected based on the ability of the cells to grow on Trp, Leu, and His dropout media supplemented with 3-aminotriazole (an inhibitor of HIS3) together with a blue colony color by β-galactosidase filter assay.

**GST Pulldown Assay—** E. coli BL21(DE3) (pGEX-GSK3β) was cultured in 3 ml of LB medium at 37 °C to the mid-log phase. Isopropylthio-β-D-galactoside was then added to a final concentration of 1 mM to induce the expression of GST fusion proteins. After culturing 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, aprtinin, and 4-(2-aminoethyl)benzenesulfonyl fluoride. The suspension was centrifuged again at 10,000 rpm for 5 min at 4 °C with a T15A22 rotor in a HITACHI CF R15 centrifuge. Glutathione-Sepharose 4B beads (20 μl) (Amersham Biosciences) were then added to the supernatant, and the mixture was incubated under shaking for 1 h at 4 °C. The beads were washed 3 times with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). After washing, the beads were added to the lysate (300 μl) prepared from E. coli lysate containing His-tagged Astrin proteins. The reaction mixture was incubated on ice for 1 h to allow binding between a GST-GSK3β protein and His-tagged Astrin proteins, including His-Astrin, His-Astrin aa 1–608, or His-Astrin aa 609–1193. The beads were subsequently washed with NETN buffer. An equal volume of 2× electrophoresis sample buffer was then added to the mixture, and proteins were extracted from the beads by heating at 95 °C for 5 min. Proteins were finally analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

**Immunoprecipitation—** HEK293 cells (1 × 10⁶ transfected with pcDNA-Astrin and pcCMV-FLAG-GSK3β were washed with phosphate-buffered saline. The lysate was prepared by adding 1 ml of immunoprecipitation 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 10 μg/ml each of leupeptin, aprtinin, and 4-(2-aminoethyl)benzenesulfonyl fluoride) to the cells. Then the lysate was centrifuged using a microcentrifuge at 10,000 × g for 20 min. The supernatant was added to anti-FLAG antibody (Roche Applied Science) at 4 °C for 1 h. Protein-A/G-agarose beads (30 μl) (Calbiochem) were added to the lysate, and the mixture was incubated with shaking for 1 h at 4 °C. The beads were then collected by centrifugation and washed three times with immunoprecipitation assay buffer. Proteins binding to the beads were eluted by adding 20 μl of 2× electrophoresis sample buffer and analyzed by immunoblotting with anti-hemagglutinin antibody. For native immunoprecipitation assays, cells were synchronized in different phases of the cell cycle and lysed by immunoprecipitation assay buffer. After immunoprecipitation, the proteins were pooled and separated by SDS-polyacrylamide gel electrophore-
Astrin and analyzed either by the anti-Astrin antibody or by the anti-hNinein antibody.

**Cell Culture, Synchronization, RNA Interference and Transfection**—HeLa cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin and streptomycin (1100 IU/ml and 100 mg/ml, respectively). For synchronization, cells were treated with 2 mM thymidine (Sigma) or 200 ng/ml nocodazole (Sigma). RNA interference mediated by duplexes of 21-nt RNAs was performed on human HeLa cells as described previously (54, 55). The following siRNA duplex oligonucleotides were used: Astrin sense 5’-CUACAGAGCCUCUGACUCUtt and anti-sense 5’-GAGAGUCAGGCUCUGUAGtt (Ambion). A scrambled siRNA duplex was used as the negative control (Ambion). For transient transfection studies, HeLa cells were seeded onto glass coverslips at a density of 8 × 10^5 cells per well in a 12-well plate. Transfection with plasmid DNA and duplex RNA was carried out using Lipofectamine 2000 (Invitrogen).

**Immunofluorescence Microscopy**—After transfection, the cells were washed with phosphate-buffered saline and fixed with 4% formaldehyde in phosphate-buffered saline for 5 min at 25 °C followed by methanol at −20 °C for 15 min. The fixed cells were probed with rabbit anti-hNinein serum (1:500, our preparation), rabbit anti-Astrin antibody (1:500, our preparation), mouse anti-Astrin antibody (1:500, our preparation), rabbit anti-Aurora A (1:500, our preparation), rabbit anti-pericentrin (1:500, Abcam), mouse anti-BubR1 (1:250, BD Biosciences), mouse anti-CENP-E (1:250, Abcam), mouse anti-α-tubulin antibody (1:1000; GTU-88, Sigma), and mouse anti-γ-tubulin antibody (1:1000; DM 1A, Sigma). DNA was stained with 4,6-diamidino-2-phenylindole (2 μg/ml). Immunofluorescent cell images were acquired using an Olympus LSM Fluoview 500 Confocal laser scanning microscope (Olympus). Images were processed with Adobe Photoshop software (Adobe Systems). All data were collected during the same session to avoid potential differences due to fading. Images were then measured for area and total pixel number for each image.
by circumscribing spindle contours and selecting that region for quantitation. Pixel number was obtained by multiplying the selected area by pixel intensity.

Preparation of Cell Extracts and Western Blot Analysis—The cell extracts were prepared in cell lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na3P2O7, 2 mM Na2VO4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science)). After incubation at 4 °C for 30 min, cellular debris was removed by centrifugation at 13,000 rpm for 10 min in an Eppendorf centrifuge. Protein concentrations were determined using the Bradford assay (Bio-Rad). Equal amounts of total lysates were used for further analyses or loaded onto a 10% SDS-PAGE, which changed the acrylamide:bis ratio from 37:1 to 100:1, and then transferred onto a polyvinylidene difluoride membrane (Millipore). The polyvinylidene difluoride membrane was then blocked with 5% bovine serum albumin, 0.1% Tween 20. Primary antibodies were incubated with the membrane at the titer of 1:1000 to 1:2500 at 4 °C for 2 h. The membranes were washed with 0.1% Tween 20 at room temperature for 10 min, repeated 3 times. Secondary antibodies against mouse IgG conjugated with horseradish peroxidase (Zymed Laboratories Inc.) were added for 1 h at room temperature followed by washing with 0.1% Tween 20 for 3 × 30 min. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (PerkinElmer Life Sciences) were added to develop the membrane.

Kinase Reaction—The His-tagged Astrin proteins were incubated with recombinant GSK3β (25 units, New England Biolabs) in the kinase reaction buffer (25 mM Tris, pH 7.2, 10 mM MgCl2, 100 μM ATP, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na3P2O7, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol) with or without [γ-32P]ATP (Amersham Biosciences) at 30 °C for 30 min followed by autoradiography. To demonstrate whether protein phosphorylation but not other post-translational modifications caused the electrophoretic mobility shift, α-phosphatase (100 units per reaction, New England Biolabs) was used to verify this finding.

RESULTS

Identification of Astrin as a Novel GSK3β-interacting Protein—To elucidate the functions of GSK3β in MT assembly, this study investigated whether GSK3β and Astrin form a complex in the spindle apparatus. A yeast two-hybrid assay was performed with recombinant activation domain-tagged GSK3β and binding domain-tagged Astrin, which showed that GSK3β interacts strongly with Astrin (Fig. 1A). Additionally, the assay was performed with deleted Astrin and revealed that an N-terminal coiled-coil region, encompassing residues 481–608, interacted with the GSK3β (Fig. 1A). On the other hand, no interaction was observed between GSK3α, a protein similar to GSK3β, and Astrin. We, therefore, conclude that Astrin binding to GSK3β is specific. To confirm the interaction between GSK3β and Astrin in vitro, recombinant GSK3β was immobilized on glutathione-Sepharose and incubated with purified hexahistidine (His6)-tagged Astrin or fragments of Astrin, amino acids 1–608 or 609–1193. His-tagged Astrin or His-tagged Astrin aa 1–608 was specifically pulled down by GSK3β in vitro (Fig. 1B). We next examined whether GSK3β and Astrin interact with each other in physiological condition. Lysates were prepared from HeLa cells at interphase or mitotic phase of the cell cycle, which were monitored using the expression of cyclins as an indicator. Immunoblot analysis revealed that both GSK3β and Astrin were coimmunoprecipitated by anti-GSK3β antibody but not by anti-GFP antibody (Fig. 1C), indicating that these two proteins interact in vivo. To compare the subcellular localization of these two proteins more directly, double indirect immunofluorescence assay was performed on HeLa cells. Confocal microscopy revealed that during interphase, GSK3β and Astrin were diffused in the cell. In mitotic cells, when cells progressed from prophase to anaphase, accumulation of GSK3β and Astrin at the spindle apparatus was observed (Fig. 2). When the cell entered the telophase, GSK3β and Astrin did not form assemblies at the midbody, although some Astrin appeared at the midbody. This suggests that complex formation between GSK3β and Astrin is enhanced in the presence of spindle apparatus.

Astrin Is a Substrate of GSK3β—To investigate whether Astrin is a substrate of GSK3β, in vitro kinase assays were performed. Assays were carried out using His-tagged Astrin, His-tagged Astrin aa 903–1193 (coiled-coil II domain), His-tagged
Astrin aa 481–902 (coiled-coil I domain), or His-tagged Astrin aa 1–481 (N-terminal head region) as substrates incubated with GSK3β in the presence of [γ-32P]ATP. Labeling results showed that His-tagged Astrin, His-tagged Astrin aa 903–1193, and His-tagged Astrin aa 1–481 were phosphorylated by GSK3β. No incorporation of 32P into the His-tagged Astrin aa 481–902 was observed (Fig. 3A). These data demonstrate that Astrin is phosphorylated by GSK3β in vitro in two regions in Astrin, from amino acids 1 to 481 and 903 to 1193. A recent phosphoproteome analysis of the human mitotic spindle also detected the presence of certain phosphorylation sites on Astrin, the phosphorylation region of which is the same as those observed in the present study (13 sites locate at N-terminal region aa 1–481 and 1 site locate at C-terminal region aa 903–1193) in the presence of two potential GSK3β phosphorylation sites (29) (Fig. 3B, cluster I and II, upper panel). These two sites, Thr-111 and Thr-937, were related to the proline directed motif, (S/T)P motif. It is also reported that GSK3β phosphorylation site consisted of a priming motif, (S/T)XXX(S/T)-p. Based on this motif algorithm, cluster III consists of a set of two Ser/Thr residues, designated Ser-974 and Thr-978. To verify that GSK3β indeed phosphorylated these predicted Astrin sites, T111A, T937A, S974A/T978A, or T937A/S974A/T978A, with candidate serine/threonine sites altered to alanines, were constructed. Compared with wild-type Astrin, the phosphorylation by GSK3β of the four mutants was reduced (Fig. 3B, middle panel). These results provide strong evidence for direct phosphorylation of Astrin by GSK3β. The effects of these mutants on GSK3β phosphorylation of Astrin were quantitated, normalized, and expressed as percentages as shown in Fig. 3B, lower panel, suggesting that GSK3β phosphorylates Astrin at these motifs.

To further determine the relevance of these phosphorylation sites, HeLa cells were transfected with either wild-type GFP-tagged Astrin or GFP-tagged Astrin 4A, which contains quadruple mutations of Thr-111/Thr-937/Ser-974/Thr-978 mutated to alanine. Meanwhile, the spindles were detected by immunostaining with α-tubulin antibody. Confocal micros-
copy revealed GFP-tagged Astrin at the spindle apparatus, overlapping with α-tubulin. The GFP-tagged Astrin 4A was still present primarily at the spindle apparatus, whereas the cell displayed an abnormal spindle morphology (Fig. 3C).

We next investigated whether the phosphorylation of Astrin could be also detected in vivo. HeLa cells were synchronized at mitosis with nocodazole. Astrin protein derived from mitotic cells migrated slower in an SDS-PAGE than did that from interphase cells (Fig. 4A, lanes 1 and 2). On the other hand, the protein from the mitotic cell migrated as fast as that from the interphase cell after the protein was treated with λ-phosphatase (Fig. 4A, lanes 3 and 4), indicating that Astrin was phosphorylated in mitotic cells. To determine that Astrin is phosphorylated by GSK3β during the mitotic phase, we cultured HeLa cells in a medium that contained LiCl or SB-415286 (30) to inhibit the function of GSK3β and reveal the level of Astrin. The data showed that mitotic Astrin exhibited slower migration in SDS-PAGE which was significantly reduced by the treatment with GSK3β inhibitors (Fig. 4B, lanes 4–6), indicating that Astrin was phosphorylated by GSK3β at mitosis. Because the inhibition of GSK3β reduced the phosphorylation of Astrin, little alteration in the total Astrin levels were seen, whereas α-tubulin was analyzed as a loading control. We also noted that LiCl or SB-415286 treatment induces cyclin B1 levels in asynchronous cells (Fig. 4B, lanes 2 and 3), suggesting that inhibiting GSK3β activity induced cell arrest in G2/M phases.

**GSK3β Kinase Activity Is Essential for the Spindle Localization of Astrin**—To assess more clearly whether Astrin phosphorylation by GSK3β is required for localization at the spindle apparatus, we used GSK3β inhibitors and analyzed the mitotic Astrin localization. The α-tubulin and γ-tubulin were used to define the spindle MTs structure and centrosome position. HeLa cells were synchronized at G2/M by thymidine-nocodazole treatment and then treated with either MeSO or GSK3β inhibitors, LiCl and SB-415286. MeSO-treated cells at the metaphase presented primarily with bipolar mitotic spindle, two spindle poles/centrosome/cell, and normal localization of Astrin or centrosomal proteins (Fig. 5A). LiCl-treated cells and SB-415286-treated cells frequently presented a spindle organization defect such as spindle organize asymmetry with the site of maximum spindle intensity within one pole (Fig. 5, B and C). We also found that inhibiting the GSK3β kinase activity affected the assembly of Astrin on the spindle MTs (Fig. 5, B and C, Astrin column). Under the same conditions the localization of centrosome component pericentrin was unaffected. The spindle association of mitotic kinase Aurora A was not significantly altered by inhibition of GSK3β activity (Fig. 5, B and C, columns of pericentrin and Aurora A). Strikingly, when GSK3β kinase was inactive, spindle organization was affected. As a result, in LiCl- and SB-415286-treated cells there was a significant increase Astrin-mislocalized cells (from % to 60%, n = 100). On the other hand, pixel intensity measurements showed that LiCl and SB-415286 all reduced of average signal intensity of Astrin on each spindle apparatus down to 35% (Fig. 5D), suggesting that the defects in spindle MTs may not interfere with spindle anchoring and nucleation but may disrupt spindle assembly.

A recent study also showed that a second pool of Astrin is most likely associated with the outer kinetochore, and in the absence of Astrin, kinetochore-microtubule attachments are impaired (24). Thus, we analyzed the level of kinetochore-bound Astrin after drug exposure. In control cells Astrin partially overlaps with the kinetochore-associated mitotic checkpoint kinase BubR1 and kinetochore-resident motor protein CENP-E. SB-415286-treated cells displayed many unattached kinetochores and fewer stable kinetochore-microtubules than control cells, and Astrin was delocalized from kinetochores (Fig. 6). Meanwhile, in SB-415286-treated cells, BubR1 and CENP-E were clearly present at the kinetochores of unaligned chromosomes, albeit CENP-E was present at reduced levels (Fig. 6). These results suggest that GSK3β activity is required for correct localization of Astrin in mitosis.

To further explore the significance of the interaction between GSK3β and Astrin, siRNA was transfected into the HeLa cells to inhibit the expression of Astrin (Fig. 7A). As shown in Fig. 7B, Astrin-depleted cells showed multipolar spindle formation. In control cells, GSK3β co-localized with Astrin at the spindle apparatus (Fig. 7C, upper panels). Furthermore, in Astrin-depleted cells, there was no detectable interference with the localization of GSK3β (Fig. 7C, lower panels). All told, these results clearly indicate that the GSK3β kinase activity is...
GSK3β Interacts with and Phosphorylates Astrin

Astrin Increases GSK3β-mediated Phosphorylation—Because Astrin interacts with GSK3β, the effect of the GSK3β-Astrin complex on the activity of GSK3β was subsequently evaluated. Our recent work showed that hNinein is a GSK3β-interacting protein, and the phosphorylation sites in hNinein are located in the C-terminal region (amino acids 1617–2090) (31, 32), which overlap with the Astrin binding region (23). Therefore, Astrin may affect GSK3β-mediated hNinein phosphorylation. To analyze the effects of Astrin on GSK3β-mediated hNinein phosphorylation, assays were carried out using recombinant hNinein (His-tagged hNinein aa 2010–2090) as a substrate. In a positive control GSK3β was found to phosphorylate hNinein (Fig. 8A, lanes 2 and 3). Interestingly, in the presence of His-tagged Astrin, the phosphorylation of hNinein was greatly increased (Fig. 8A, lane I compare with lanes 2 and 3), indicating that Astrin enhances the kinase activity of GSK3β. Therefore, we also tested the capacity of Astrin to increase GSK3β-mediated phosphorylation of Axin and Tau (Fig. 8, B and C). As expected, in the presence of His-tagged Astrin, phosphorylation at both Axin and Tau increased significantly. On the other hand, when Aurora A was used under the same conditions, there was no significant difference in Aurora A activity either in the absence or presence of Astrin (Fig. 8D). We then tested the requirement of the interaction domain in Astrin for enhancing the kinase activity of GSK3β. Only His-tagged Astrin and His-tagged Astrin aa 479–1193 facilitated the GSK3β-mediated phosphorylation of hNinein (Fig. 8E). These data clearly demonstrate that Astrin specifically enhances phosphorylation mediated by GSK3β.

Because Astrin protein binds to GSK3β and facilitates GSK3β activity in vitro, we next examined the effects of Astrin on GSK3β-mediated phosphorylation in situ. HEK293 cells were transiently cotransfected with GFP-tagged Tau and FLAG-tagged GSK3β in the presence or absence of Myc-tagged Astrin. Phosphorylation of Tau was examined by immunoblotting with phospho-specific antibodies (Fig. 9A). As expected, exogenous expression of Astrin resulted in increased phosphorylation at the unprimed PHF-1 epitope (phospho-Ser-396/404). In contrast, when Astrin was expressed, GSK3β phosphorylation of Tau at the primed AT180 epitope (phospho-Thr-231) was not significantly different, indicating a selective facilitation of GSK3β-mediated Tau phosphorylation by Astrin. When treated with 40 mM LiCl, phosphorylation of Tau was dramatically reduced. The effects of Astrin on GSK3β phosphorylation of Tau at the unprimed site PHF-1 (phospho-Ser-396/404) and the primed site AT180 (phospho-Thr-231) were quantitated, normalized to the levels of GFP-Tau/FLAG GSK3β, and expressed as percentages of the Tau-only condition. Quantitation revealed that Astrin increased the GSK3β-mediated Tau PHF-1 site phosphorylation by almost 4-fold (Fig. 9B). Together, these data clearly demonstrate that Astrin facilitates GSK3β phosphorylation of an unprimed epitope in Tau while not affecting the phosphorylation of a primed epitope.

FIGURE 5. GSK3β activity is required for correctly localization of Astrin. HeLa cells were arrested in M phase by thymidine–nocodazole (Noc) block followed by a 1.5-h treatment with Me2SO (DMSO; A), 40 mM LiCl (B), or 30 μM SB-415286 (C), fixed, and analyzed by immunofluorescence microscopy. Cells were stained for γ-tubulin or α-tubulin (red) and either Astrin, Pericentin, or Aurora-A (green) as indicated. Note the presence of abnormal spindles in GSK3β-inhibiting cells (B and C). The scale bar represents 10 μm. Schematic representations of the experimental procedures are depicted below the image panels. D, quantification in terms of the percentage of proteins localized in cells exposed to Me2SO (control), LiCl, or SB-415286. Me2SO, n = 200; LiCl, n = 200; SB-415286, n = 129; error bar = S.E.; **, p < 0.01.

essential for the spindle assembly of Astrin. Conversely, the spindle association of GSK3β does not depend on the presence of Astrin.
It appears that Astrin enhances GSK3β activity through both direct and indirect mechanisms. To investigate these possibilities, as shown in Fig. 9C, both full-length Astrin and amino acids 479–1193 of Astrin were sufficient to facilitate the GSK3β-mediated phosphorylation of Tau compared with what was observed with GSK3β alone. However, amino acids 609–1193 were inefficient at activating GSK3β; this segment was not a proficient interaction protein (Fig. 9C). The quantitated percentages are schematized in Fig. 9D. Overall, these findings clearly indicate that Astrin directly enhances GSK3β activity.

**DISCUSSION**

In this report we describe the identification of the spindle-associated protein Astrin as an interaction partner and candidate substrate for GSK3β. In particular, we show that the association of Astrin with spindle MT and kinetochore requires GSK3β kinase activity. Our data also reveal that Astrin facilitates GSK3β-mediated phosphorylation. We, therefore, propose that GSK3β and Astrin are linked at the spindle apparatus during mitosis.

**GSK3β-Astrin Complex Exclusively Localizes on Spindle Microtubules**—Etienne-Manneville and Hall (17) demonstrated that GSK3β is involved in the polarized anchoring of microtubules at the cell periphery. Recently studies also demonstrated that inhibition of GSK3β causes aberrant mitosis such as extended astral microtubules and chromosome misalignment (18, 19). Until now, however, no clear candidate substrate for GSK3β has emerged to establish a direct relationship between GSK3β and spindle MTs components. Here, we show that the N-terminal half of coiled-coil I domain, residues 479–608, of Astrin interacts directly with the GSK3β (Fig. 1). Indeed, the C-terminal region of Astrin is the primary determinant for targeting Astrin to spindles (21). These interactions suggest that GSK3β and Astrin contact directly, and then the GSK3β-Astrin complex may be recruited to the spindle apparatus through Astrin. However, our results cannot exclude the possibility that the association of the GSK3β-Astrin complex with spindles may involve more complex intermolecular interactions co-mediating the dynamic interaction between GSK3β and spindle MTs.

Previous studies showed that both GSK3β and Astrin are localized to the spindle apparatus (18–21, 23). In this study, we show that GSK3β and Astrin are colocalized in mitotic cells on the spindle apparatus from the prophase to the anaphase. It should be noted that the assembly of a functional bipolar mitotic spindle requires an exquisite regulation of microtubule behavior in time and space. Thus, the GSK3β-Astrin complex specific association with spindles would suggest a functional
GSK3β Interacts with and Phosphorylates Astrin

The GSK3β-mediated phosphorylation raises questions with respect to the physiological significance of Astrin. It appears that the spindle and the kinetochore association of Astrin may be dependent on phosphorylation by GSK3β (Figs. 5 and 6). However, it is still unclear whether the Astrin 4A mutant does not mislocalize from the spindle MTs but affects the spindle morphology (Fig. 3C). It is attractive to postulate that these sites might affect spindle assembly through regulation of the Astrin function and/or interaction with other spindle-associated proteins. The other possibility is that phosphorylation at Thr-111/Thr-937/Ser-974/Thr-978 may be not completely responsible for regulation of Astrin localization. In future studies it will be important to map more GSK3β phosphorylation sites within Astrin and analyze the phosphorylation state of each site through the cell cycle.

It remains to be clarified whether Astrin is phosphorylated at the spindle or translocated to the spindle after phosphorylation. We show here that GSK3β and Astrin are not present at the centrosome during the interphase, and GSK3β activity is required for Astrin hyperphosphorylation in the mitotic phase. Inhibition of GSK3β activity during mitosis affects polarized anchoring of spindle MTs and their length. Phospho-Astrin may be translocated to the spindle by an unknown mechanism after GSK3β phosphorylation at the G2/M transition. On the other hand we cannot exclude the possibility that GSK3β indirectly stimulates the phosphorylation of Astrin at spindles by activating another kinase, such as Aurora A and Plk1.

The spindle assembly ensures high fidelity mitotic chromosome segregation. Recent studies indicate that Cdk1, protein kinase A, Bub1 kinase, Polo-like kinase, Nek kinase, and the proteins in the Aurora family may be involved in regulating the centrosome cycle and formation of the mitotic spindle (32–36). Dysregulation of these kinases by overexpression or inhibition results in centrosome defects, spindle aberrations, chromosome instability, and tumor progression in cancerous conditions. GSK3β is the main kinase involved in the Wnt canonical pathway and is named from the substrate glycogen synthase. It can phosphorylate more than 40 proteins, including over a dozen transcription factors and microtubule-associated proteins (3, 37–39). Evidence based on inhibition of GSK3β in mitosis suggests that GSK3β may be a controlling spindle factor that affects protein localization, spindle MTs formation, and radial MTs array (Figs. 5 and 6). This is consistent with several studies showing a role for GSK3β in chromosomal alignment and microtubule organization at the centrosome (18, 19, 28, 40, 41). It is, therefore, reasonable that GSK3β kinase activity is indeed as an important factor in mitotic spindle regulation.

Chromosome segregation is not only mediated by spindle microtubules but also by microtubule-kinetochore attachment (42–44). In SB-415286-treated cells, BubR1 persisted at metaphase kinetochores with the unattached kinetochores staining stronger (Fig. 6), which is consistent with previous observations by Tighe et al. (19). We also found that Astrin and CENP-E levels were significantly reduced at kinetochores (Fig. 6). These phenomena agree with the observation that Astrin-depleted cells display many unattached kinetochores with the CENP-E delocalized from the kinetochore (24). These results suggest that GSK3β kinase activity is involved in kinetochore recruit-

role in spindle organization. Another aspect of Astrin is its association with kinetochore, suggesting that Astrin is a GSK3β target with an important role in chromosomal alignment at the metaphase (Fig. 2). In contrast, the midbody was strongly positive for Astrin but weakly stained by anti-GSK3 antibodies, suggesting differential subcellular localization (Fig. 2, Telophase). Therefore, Astrin may be regulated by other kinases rather than GSK3β at the midbody, such as Plk1 and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK).

Astrin Localization onto the Spindle and the Kinetochores May Be Dependent on Phosphorylation by GSK3β—The kinase responsible for Astrin phosphorylation has not yet been identified. Our results clearly demonstrate that Astrin is a candidate substrate of GSK3β (Figs. 3 and 4). On the other hand, two identified sites of phosphorylation are followed by prolines, a motif for proline-directed kinase. The other possibility is that another critical kinase is involved in the regulating phosphorylation, such as the Cdk families and mitogen-activated protein kinase. In addition, using phosphoproteome analysis, Nousiainen et al. (29) suggested the presence of two potential in vivo Cdc2 kinase phosphorylation sites and five potential in vivo extracellular signal-regulated kinase phosphorylation sites within the N terminus of Astrin. We speculate whether these kinases may also play a role in the phosphorylation of Astrin.
Astrin Directly and Specifically Activates GSK3β on Unprimed Sites—Previous studies have shown that GSK3β activity can be regulated by its interaction with various proteins. For example, FRAT-1 (45), low density lipoprotein receptor-related protein 6 (46), and GSKIP (47) directly interact with GSK3β, which results in an attenuation of GSK3 activity. In this study we found that GSK3β-mediated phosphorylation of various substrates, such as hNinein, Axin, and Tau, is increased in the presence of recombinant Astrin in vitro, suggesting that Astrin facilitates GSK3β activity (Fig. 8). This is the first report to demonstrate that a MTs-associated protein binds to GSK3β and is selectively phosphorylated by GSK3β.

Tau is a well characterized substrate of GSK3β both in situ and in vitro. Recently, it has been demonstrated that Tau is modified by GSK3β, including both primed (S/T)XXX(S/T)-p and unprimed (S/T)P motifs (3). The phosphorylation state of Tau is directly correlated to its ability to bind and stabilize microtubules. The importance of phosphorylation at a primed site, such as the AT180 site (Thr-231), is in regulating Tau-microtubule interactions (48). Indeed, the increased phosphorylation of unprimed GSK3β sites in Tau, such as the PHF-1 site (Ser-396), may be pathological (49). Our data show that Astrin facilitates the GSK3β-mediated phosphorylation of Tau at unprimed sites (Fig. 9), which implies that Astrin may play a role in the Tau pathological phosphorylation by GSK3β in Alzheimer’s disease. In mitotic cells, however, regulation of PHF-1 phosphorylation changes dramatically. This type of Tau phosphorylation, referred to as “mitotic Tau phosphorylation,” has been extensively studied (50–53), and some “mitotic Tau PHF-1 phosphorylation” may also contribute to spindle organization. Taken together, our data indicate that Astrin may modulate both physiological and pathological phosphorylation by GSK3β.

In summary, we identified the spindle-associated protein Astrin as a novel interaction partner and candidate substrate of GSK3β. The data strongly suggest that GSK3β acts as a real regulator on spindle MTs formation, assembly, and microtubule-kinetochore attachment. In particular, we show that the association of Astrin with spindle MTs and kinetochores may be dependent on GSK3β kinase activity. Moreover, upon Astrin binding, the GSK3β-Astrin complex specifically increases GSK3β-mediated phosphorylation on unprimed sites, suggesting that this molecular mechanism is vital for spindle regulation.

Acknowledgment—We thank Professor Shih-Tung Liu for reading and suggestions.

REFERENCES

1. Ali, A., Hoeflich, K. P., and Woodgett, J. R. (2001) Chem. Rev. 101, 2527–2540
2. Cohen, P., and Frame, S. (2001) Nat. Rev. Mol. Cell Biol. 2, 769–776
3. Jose, R. S., and Johnson, G. V. (2004) Trends Biochem. Sci. 29, 95–102
4. Jose, R. S., Yuskaitis, C. J., and Beurel, E. (2007) Neurochem. Res. 32, 577–595
5. Doble, B. W., and Woodgett, J. R. (2003) J. Cell Sci. 116, 1175–1186
6. Wang, Q. M., Park, I. K., Fiol, C. J., Roach, P. J., and DePaoli-Roach, A. A. (1994) Biochemistry 33, 143–147
7. Phiel, C. J., Wilson, C. A., Lee, V. M., and Klein, P. S. (2003) Nature 423, 433–439
8. Liang, M. H., and Chiang, D. M. (2007) J. Biol. Chem. 282, 3904–3917
9. Liang, M. H., and Chiang, D. M. (2006) J. Biol. Chem. 281, 30479–30484
10. Sutherland, C., Leighton, I. A., and Cohen, P. (1993) Biochem. J. 296, 15–19
11. Cohen, P. (1999) Philos. Trans. R. Soc. Lond. B. Biol. Sci. 354, 485–495
12. Fiol, C. J., Mahrenholz, A. M., Wang, Y., Roeke, R. W., and Roach, P. J. (1987) J. Biol. Chem. 262, 14042–14048
13. Song, I. S., and Yang, S. D. (1995) J. Protein Chem. 14, 95–105
GSK3β Interacts with and Phosphorylates Astrin

14. Cole, A. R., Causeret, F., Yaeirgi, G., Hastie, C. J., McLauchlan, H., McManus, E. I., Hernandez, F., Eickholt, B. J., Nikolic, M., and Sutherland, C. (2006) J. Biol. Chem. 281, 16591–16598
15. Goold, R. G., and Gordon-Weeks, P. R. (2005) Mol. Cell. Neurosci. 28, 524–534
16. Zhou, F. Q., and Snider, W. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9578–9582
17. Cognen, P., Culbert, A. A., Cross, D. A., Corcoran, S. L., Yates, J. W., Pearce, N. J., Rausch, O. L., Murphy, G. J., Carter, P. S., Roxbbee Cox, L., Mills, D., Brown, M. J., Haigh, D., Ward, R. W., Smith, D. G., Murray, K. I., Reith, A. D., and Holder, J. C. (2000) Biochem. Biophys. Res. Commun. 287, 595–602
18. Chien, C. T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9578–9582
19. Dogiel, A. A., Kelleher, R. J., Sergeant, N., Delacourte, A., Vilain, J. P., and Buee, L. (2002) J. Cell Biol. 156, 1145–1157
20. Abraha, A., Ghoshal, N., Gamblin, T. C., Cryns, V., Berry, R. W., Kuret, J., and Binder, L. I. (2000) J. Cell Sci. 113, 3737–3745
21. Pope, W. B., Lambert, M. P., Leybold, B., Seupaul, R., Sletten, L., Krafft, G., and Klein, W. L. (1994) Exp. Neurol. 126, 185–194
22. Preuss, U., Doring, F., Illenberger, S., and Mandelkow, E. M. (1995) Mol. Biol. Cell 6, 1397–1410
23. Vincent, I., Rosado, M., and Davies, P. (1996) J. Cell Biol. 132, 413–425
24. Delobel, P., Flamant, S., Hamdane, M., Maillot, C., Sambo, A. V., Begard, S., Sergeant, N., Delacourte, A., Vilain, J. P., and Buee, L. (2000) J. Neurochem. 83, 412–420
25. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Nature 411, 494–498
26. Harborth, J., Elbashir, S. M., Bechert, K., Tuschl, T., and Weber, K. (2001) J. Cell Sci. 114, 4557–4565
27. Zhu, L. (1997)
28. Huang, P., Senga, T., and Hamaguchi, M. (2007) BMC Cell Biol. 8, 34
29. Nousiainen, M., Sillje, H. H., Sauer, G., Nigg, E. A., and Korner, R. (2006) Exp. Cell Res. 313, 1710–1721
30. Coghlan, M. P., Culbert, A. A., Cross, D. A., Corcoran, S. L., Yates, J. W., Lin, W. C., and Yang, Y. C. (2001) Biochem. Biophys. Res. Commun. 287, 116–121
31. Chen, C. H., Howng, S. L., Cheng, T. S., Hsiao, Y. L., Lieu, A. S., Loh, J. H., Kwong, S. L., Lin, C. C., Hsu, C. M., Lee, C. I., Yu, C. T., Huang, C. Y., Howg, S. L., and Hong, Y. R. (2007) Exp. Cell Res. 313, 1710–1721
32. Tighe, A., Ray-Sinha, A., Staples, O. D., and Taylor, S. S. (2007) BMC Cell Biol. 8, 34
33. Sanchez, C., Perez, M., and Avila, J. (2000) J. Cell Biol. 150, 1119–1129
34. Doxsey, S., Zimmerman, W., and Mikule, K. (2005) Trends Cell Biol. 15, 303–311
35. Azimzadeh, I., and Bornens, M. (2007) J. Cell Sci. 120, 2139–2142
36. Malumbres, M., and Barbacid, M. (2007) Curr. Opin. Genet. Dev. 17, 60–65
37. Lovestone, S., Hartley, C. L., Pearce, J., and Anderton, B. H. (1996) Neuroscience 73, 1145–1157