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A novel ninein-interaction protein, CGI-99, blocks ninein phosphorylation by GSK3β and is highly expressed in brain tumors

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Abstract To explore more hNinein interacting proteins, the yeast two-hybrid screening using ninein C-terminal domain as bait protein was performed. One novel gene, *CGI-99*, was demonstrated to associate with hNinein in the yeast two-hybrid method and in vitro GST pull-down assay. Molecular characterization also showed that CGI-99 possessed a transcriptional activity at the N-terminal. In addition, CGI-99 formed a dimer with the C-terminal, which overlapped with hNinein binding site. In kinase assay, CGI-99 binds to hNinein and completely blocks the phosphorylation of hNinein by GSK3β. Moreover, CGI-99 was highly expressed in all brain tumors which is in agreement with the Northern blot analysis. Taken together, we have isolated a novel protein CGI-99, which may be involved in the functional regulation of human ninein in the centrosome structure and may also be important in brain development and tumorigenesis.
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Keywords: Ninein; Centrosome; CGI-99; GSK3β; Brain tumor

1. Introduction

The centrosome plays key roles in the formation of the mitotic spindle, cell polarity and cell locomotion. In a typical somatic cell, the centrosome is composed of a pair of centrioles that are surrounded by a mass of amorphous pericentriolar material (PCM). The recent identification of molecular components shows that PCM may be involved in the formation of the complex of γ-tubulin [1,2], centrin [3–5], pericentrin [6] and ninein [7–13,12,14], which are organized into a highly ordered lattice [13,15,16]. More recently, the centrosomal-associated protein, hNinein, has been identified as a microtubules minus end capping [10], centrioles position [13,12], centrosome maturation [15,16] and anchoring protein [11,12], but the underlying structure and physiological function are not well understood.

Recently, a growing body of several structurally distinct protein kinases (PKAs) has been found to be localized at the centrosome [14,17–21]. The physiological roles of polo-like kinase 1 and NeK2 in the centrosome integrity and separation are demonstrated, respectively [17,20,21]. In addition, aurora A (AIK) and cAMP-dependent PKA have been suggested to have possible involvement in chromosome missegregation, aneuploidy and genomic instability [19–21]. Our previous study was also to show that human ninein interacts with GSK3β [22], but the C-terminal fragment of hNinein (1617–1931 aa) failed to serve as a substrate [14].

To explore more hNinein interacting proteins, the yeast two-hybrid screening using hNinein C-terminal domain (1617–2090 aa) as a bait protein was performed. One novel gene, *CGI-99*, was demonstrated to associate with hNinein in the yeast two-hybrid method and in vitro GST pull-down assay. We also show that CGI-99 binds to hNinein and prevents the phosphorylation of hNinein by GSK3β. Moreover, CGI-99 is highly expressed in all brain tumors. Our data suggest that CGI-99 may not only participate in the centrosome architecture but also contribute to brain development and tumorigenesis as well.

2. Materials and methods

2.1. Cloning and DNA sequencing

To construct plasmids, pET-32a-CGI-99 for expressing His-tagged CGI-99 in *Escherichia coli* BL21(DE3), DNA fragments encoding the CGI-99 were amplified by PCR with the Taq polymerase (TaKaRa), using primers containing sense sequences, 5′-CGGAATTCATGTTCCGACGCAAGTTGACGG, and antisense sequences, 5′-CCGCTCGAGTCATCTTCCAACTTTTCCC. The PCR fragments were then inserted into the *Eco*RI–*Xho*I sites in pET-32a (Novagen) or pGEX-KG vectors (Novagen). A full-length CGI-99 was constructed to pEGFPC2 vector (Clontech) and were fused at the restriction sites *Eco*RI and *Xho*I. The N-terminal (1–150 aa) and C-terminal (151–244 aa) of CGI-99 were amplified by PCR. These amplified fragments were digested by restriction enzyme and they were constructed into pACT2 and pAS2-1 vectors. The nucleotide sequencing was performed by ABI PRISM™ 3730 Genetic Analyzer (Perkin–Elmer).

2.2. Yeast two-hybrid system

Standard techniques were used for the yeast two-hybrid screening [23–25]. Briefly, the CCII domain of human ninein (1617–2090 aa) was cloned in frame with the Gal4 DNA-binding domain in the pAS2-1 vector (MARCHMAKER Two-Hybrid System 2, Clontech) to yield pAS2-1-CCII bait plasmid. A human testis cDNA library was screened by co-transforming yeast YRG-2 (Stratagene) with pAS2-1-CCII bait

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plasmid DNA and human adult testis library plasmid DNA (Clontech). The positive clones have the ability to grow on Trp, Leu, and His dropout media supplemented with 3-aminotriazole (3-AT, and an inhibitor of HIS3) and turn blue in β-galactosidase filter assay.

2.3. Cell culture, transfections and indirect immunofluorescence

HeLa cells were grown at 37 °C in DMEM supplemented with 10% FCS and penicillin–streptomycin (100 IU/ml). For transient transfection studies, HeLa cells were seeded onto glass coverslips at a density of 0.7×10^5 cells per 24-well plate. DNA was transfected with 1 μg into HeLa cells, using Lipofectamine plus reagent (Life Technologies). After 24 h, cells were treated with a final concentration of 100 ng/ml nocodazole (drugs purchased from Sigma) for 15 h. Then, the cells were fixed in cold methanol for 20 min and immunostained as described [26]. The fixed cells were probed with anti-α-tubulin polyclonal antibody. The secondary antibodies were rhodamine-conjugated goat anti-rabbit Fab fragment (1:300; Santa Cruz) and DNA was stained with DAPI (Roche).

2.4. Northern blot analysis

Human Northern blot containing poly(A^+)-RNAs from adult tissues, including the heart, brain, placenta, lung, liver, skeletal muscle,

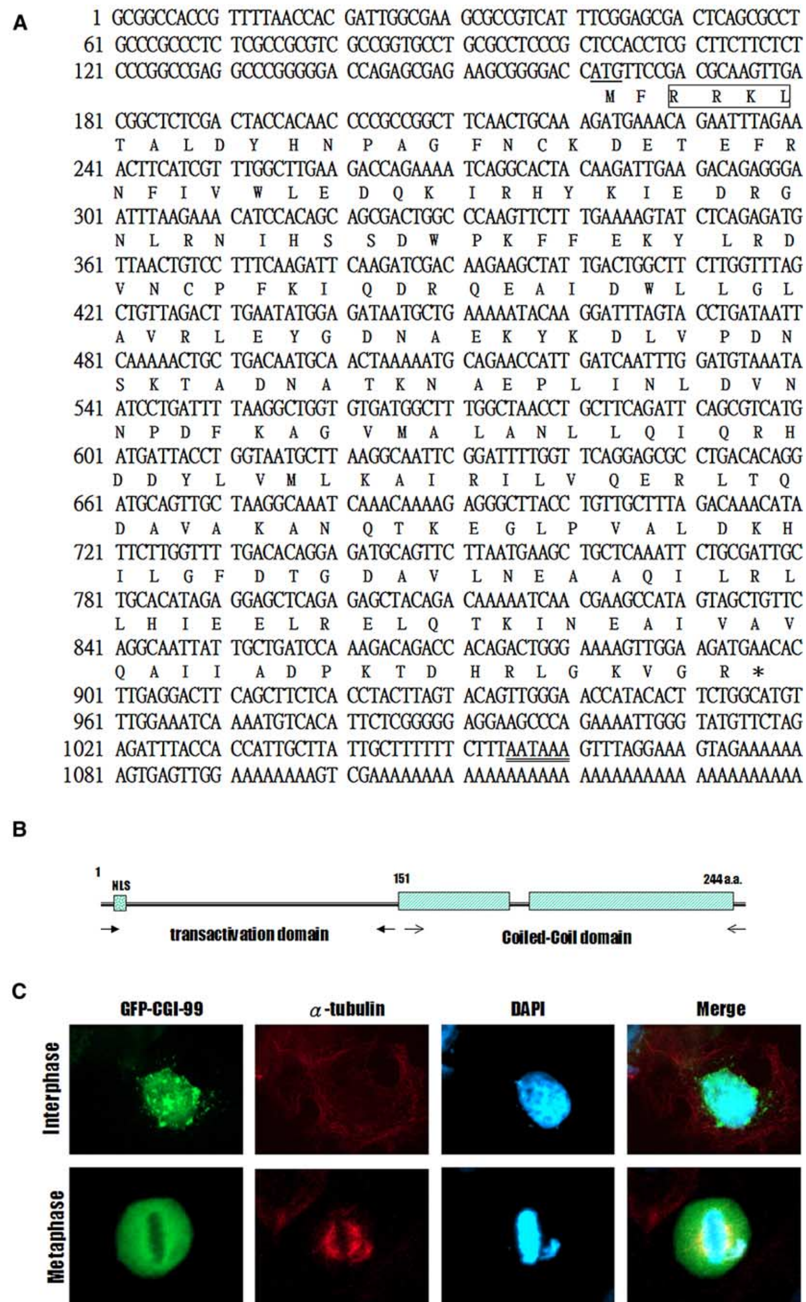


Fig. 1. Nucleotide and deduced amino acid sequences of CGI-99. (A) The location of the predicted nuclear location signal (NLS) is boxed. The AATAAA polyadenylation signal is double-underlined. The nucleotide sequences for CGI-99 have been assigned to the GenBank database as Accession No. NP_057123. (B) Coiled-coil regions of CGI-99 C-terminal, predicated by the program of Lupas et al. [30]. Note that N-terminal of CGI-99 contains an activation domain (see Fig. 3B). (C) Localization of CGI-99. CGI-99 was localized to the nuclear compartment with some labelled foci in the perinuclear region of the cell in interphase (upper panel), in metaphase (lower panel). Noted to compare GFP-CGI-99 and α-tubulin in a representative interphase or metaphase cell.

kidney and pancreas or from human fetal tissues, including the brain, lung, liver and kidney were obtained 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) and 1% SDS, salmon sperm DNA (100 μg/ml) with [α-^{32}P]dCTP-labeled cDNA probe. The probe used was a 0.73-kb cDNA full-length of CGI-99. The blots were rinsed twice in 2× SSC, 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.

2.5. RT-PCR

The cDNAs from the human brain were obtained from Clontech and used as templates for tissue-specific PCR. Total RNA was extracted from brain tumors by means of the acid guanidinium phenol/chloroform method and cDNA was synthesized with molonry murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA). In order to identify CGI-99 expression in different brain tumors, primers CGI-99-5′(5′-ATGTTCCGACGCAAGTTGACGG-3′) and CGI-99-3′(5′-TCATCTTCCAACTTTTCCC-3′), corresponding to the 732-bp region, were used for detection of the CGI-99. The PCR mixture contained 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 mM dNTP and 2 μM of each primer with 1 U of ExTag polymerase (TaKaRa). The PCR involved denaturation at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min for a total of 35 cycles. After PCR, the total product was electrophoresed on a 1.2% polyacrylamide gel and stained by ethidium bromide. Gels were photographed using Polaroid film and the intensity of CGI-99 was measured by means of a densitometer.

2.6. Western blot analysis

For western blot analysis, *E. coli* were harvested and washed once in PBS. Later, cells were resuspended in cell lysate buffer (20 mM PIPES, pH 7.2, 100 mM NaCl, 1 mM PMSF, 1 mM EDTA, 0.1% CHAPS and 10% sucrose). Samples were left for 30 min on ice and centrifuged at 14 000 rpm for 30 min at 4 °C. The supernatant was removed into a fresh centrifuge tube, the protein sample buffer was added, and the sample was heated to 95 °C for 5 min following analysis on a 12% SDS–PAGE as previously described [27]. Proteins were transferred to PVDF and incubated for 1 h in blocking buffer (5% in PBS/0.1% Tween 20). His or GST polyclonal antibody incubations were carried out first in blocking buffer for 1 h at room temperature and second antibody using HRP-conjugated anti-rabbit antibodies for another hour.

2.7. GST pull-down assay

E. coli BL21(DE3) (pGEX-CGI-99, pGEX-4T1 vector) 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, Rockford, IL), containing 10 μl leupeptin, aprotinin 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 CFR15 centrifuge. Glutathione–Sepharose 4B beads (20 μl) (Amersham Pharmacia Biotech) were then added to the supernatant and the mixture was incubated under 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 washing, the beads were added to the lysate (300 μl) prepared from *E. coli* lysate containing His-tagged C-terminal hNinein. The reaction mixture was incubated on ice for 1 h to allow the binding between a GST-fusion protein, including GST-CGI-99, His-tagged C-terminal hNinein (1617–2090 aa) and His-tagged C-terminal hNinein (1617–1931 aa) as described [14]. 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 at 95 °C for 5 min. Proteins were finally analyzed by SDS–PAGE and immunoblotting.

2.8. In vitro kinase assays

Kinase reaction was carried out [17,18]. Briefly, the hNinein C-terminal protein was purified and incubated with GSK3β kinases in kinase buffer (100 mM HEPES, pH 7.4, 1 mM dithiothreitol, 10% glycerol, 0.4 mM ATP, 80 mM $MgCl_2$ and 10 μCi of [γ-^{32}P]ATP (Amersham, 3000 Ci/mM)). The assays were carried out for 20 min at 30 °C. Reactions were stopped by the addition of 2× sample buffer and heated at 95 °C for 5 min, followed by SDS–PAGE and detection by autoradiography.

3. Results and discussion

3.1. Molecular cloning of CGI-99 and nuclear localization

In this study, we surveyed hNinein C-terminal domain (1617–2090 aa) interacting proteins from a human testis cDNA library (Clontech) using the yeast two-hybrid system. One of these interacting proteins was CGI-99, which sequence conservation was found in different species [28,29]. The cDNA sequence contained an open reading frame of 732 bp encoding a polypeptide of 244 amino acids with a predicted molecular mass of 28 113 Da ($pI = 6.49$) (Fig. 1A). As illustrated in Fig. 1B, C-terminal of the CGI-99 sequence was predicted to contain coiled-coil structure based on the algorithm by Lupas et al. [30].

To identify the subcellular localization of CGI-99, HeLa cells expressing the GFP-CGI-99 fusion were examined by fluorescence microscopy. A computer-assisted search for the motifs presented in CGI-99 protein found a nuclear localization signal, suggesting that CGI-99 could be a nuclear protein. Indeed, ectopically expressed GFP epitope-tagged CGI-99 was localized to the nuclear compartment with some labelled foci in the perinuclear region of the cell (Fig. 1C, upper panel); however, CGI-99 may be localized at the spindle body during metaphase (Fig. 1C, lower panel).

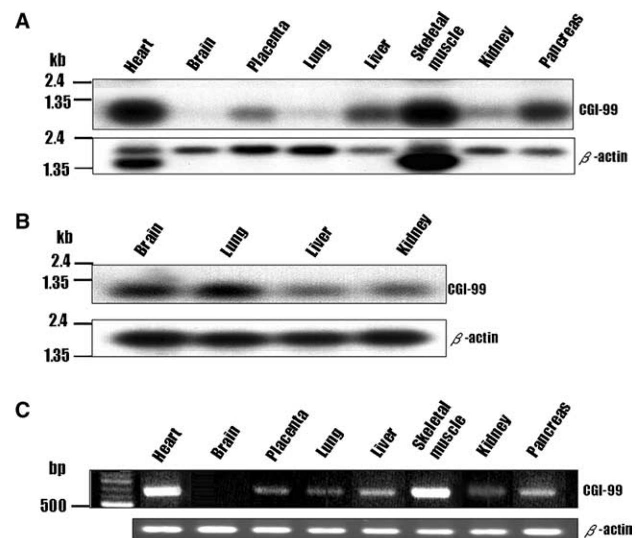


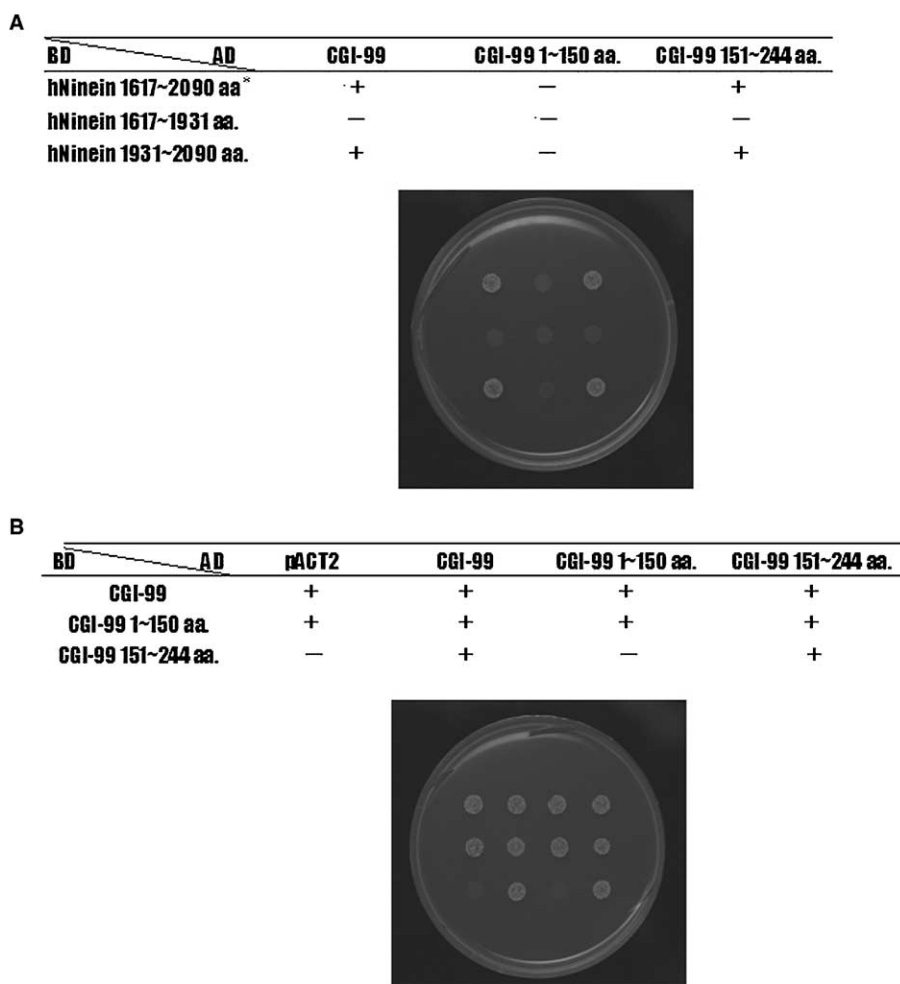
Fig. 2. Northern blot analysis of CGI-99 expression in various human tissues. The membrane contained ~2 μg of poly(A^+) mRNA from each tissue. Hybridization was done using [α-^{32}P]-labelled cDNA probe for the full-length CGI-99 and the human β-actin as a control. (A) Adult tissues including the heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas are indicated. (B) Fetal tissues including the brain, lung, liver and kidney. (C) Quantitative PCR analysis of CGI-99 expression in different human tissues. PCR primers (sense sequences, 5′-ATGTTCCGACGCAAGTTGACGG and antisense sequences, 5′-TCATCTTCCAACTTTTCCC) specific for CGI-99 full-length expected product size is 732-bp (upper panel); β-actin as positive control (lower panel). M, 100 bp marker.

3.2. Expression of CGI-99 in various human tissues

By Northern blot analysis, a 1.1-kb transcript was found in almost all adult tissues examined with a relatively higher expression level in the heart and skeletal muscle (Fig. 2A). In brain, however, the transcript was absent (Fig. 2A, lane 2). Interestingly, the transcript was expressed more distinctly in the fetal brain and lung than in the adult brain and lung (Fig. 2B), suggesting that this protein may play a role during brain and lung development. The CGI-99 expression level in adult tissues was further confirmed by RT-PCR (Fig. 2C).

3.3. CGI-99 interacts with hNinein in vivo and in vitro

Since large parts of the hNinein sequence contain a coiled-coil structure [11]; in order to determine whether coiled-coil domain was involved in this interaction, we then tested the interaction between hNinein and CGI-99 using the yeast two-hybrid system. The data show that C-terminal of the CGI-99 coiled-coil structure (151–244 aa) specifically interacts with hNinein in a very C-terminal coiled-coil domain (1931–2090 aa), but not the previously reported dimerization domain (1617–1931 aa) (Fig. 3A). Molecular characterization also showed that CGI-99 possessed a transcriptional activity at the N-terminal residues (1–150 aa) (Figs. 3B and 1B). To further confirm such protein–protein interaction, CGI-99 and hNinein were overexpressed to carry out in vitro binding assay. The cDNA of CGI-99 and two hNinein C-terminal regions (residues 1617–1931 and 1617–2090 aa) were cloned into pET vector to produce a His-Tag fusion protein, and the full length of CGI-99 cDNA was cloned into a pGEX-KG vector to generate a GST fusion protein. Purified His-Tag-hNinein (1617–1931 aa), His-Tag-hNinein (1617–2090 aa), His-Tag-CGI-99; and GST-CGI-99 fusion proteins were analyzed by SDS–PAGE (Fig. 4A, left panel) and Western blotting (Fig. 4A, right panel). The results of in vitro GST pull-down assay showed that GST-CGI-99 binds to His-Tag-hNinein (1617–2090 aa), but not His-Tag-hNinein (1617–1931 aa) (Fig. 4B, compare lane 6 to lane 4). Moreover, His-Tag-CGI-99 and GST-CGI-99 fusion proteins were pulled down as detected by Western blotting (Fig. 4C, lane 4), suggesting that CGI-99 indeed forms a dimer. These data are consistent with our above observation in the yeast two-hybrid screening (Fig. 3). Furthermore, co-expressed hNinein and CGI-99 in HeLa cells also show tht they are partially co-localized in the centrosome during interphase (Fig. 4D). Although CGI-99 and



A

BD \ AD	CGI-99	CGI-99 1~150 aa.	CGI-99 151~244 aa.
hNinein 1617~2090 aa*	+	–	+
hNinein 1617~1931 aa.	–	–	–
hNinein 1931~2090 aa.	+	–	+

B

BD \ AD	pACT2	CGI-99	CGI-99 1~150 aa.	CGI-99 151~244 aa.
CGI-99	+	+	+	+
CGI-99 1~150 aa.	+	+	+	+
CGI-99 151~244 aa.	–	+	–	+

Fig. 3. Yeast two-hybrid assay showing the interaction of CGI-99 and hNinein. (A) Interaction of CGI-99 and hNinein. (B) Dimerization of CGI-99. "*" indicates that original hNinein C-terminal domain (1617–2090 aa) was used as a bait. "+" indicates positive interaction. "–" indicates negative interaction. Numbers indicate the portion of amino acid residues in CGI-99 or hNinein. Yeast containing plasmids were spotted on Trp, Leu, and His dropout media supplemented with 3-AT plates. Growth indicates a positive interaction.

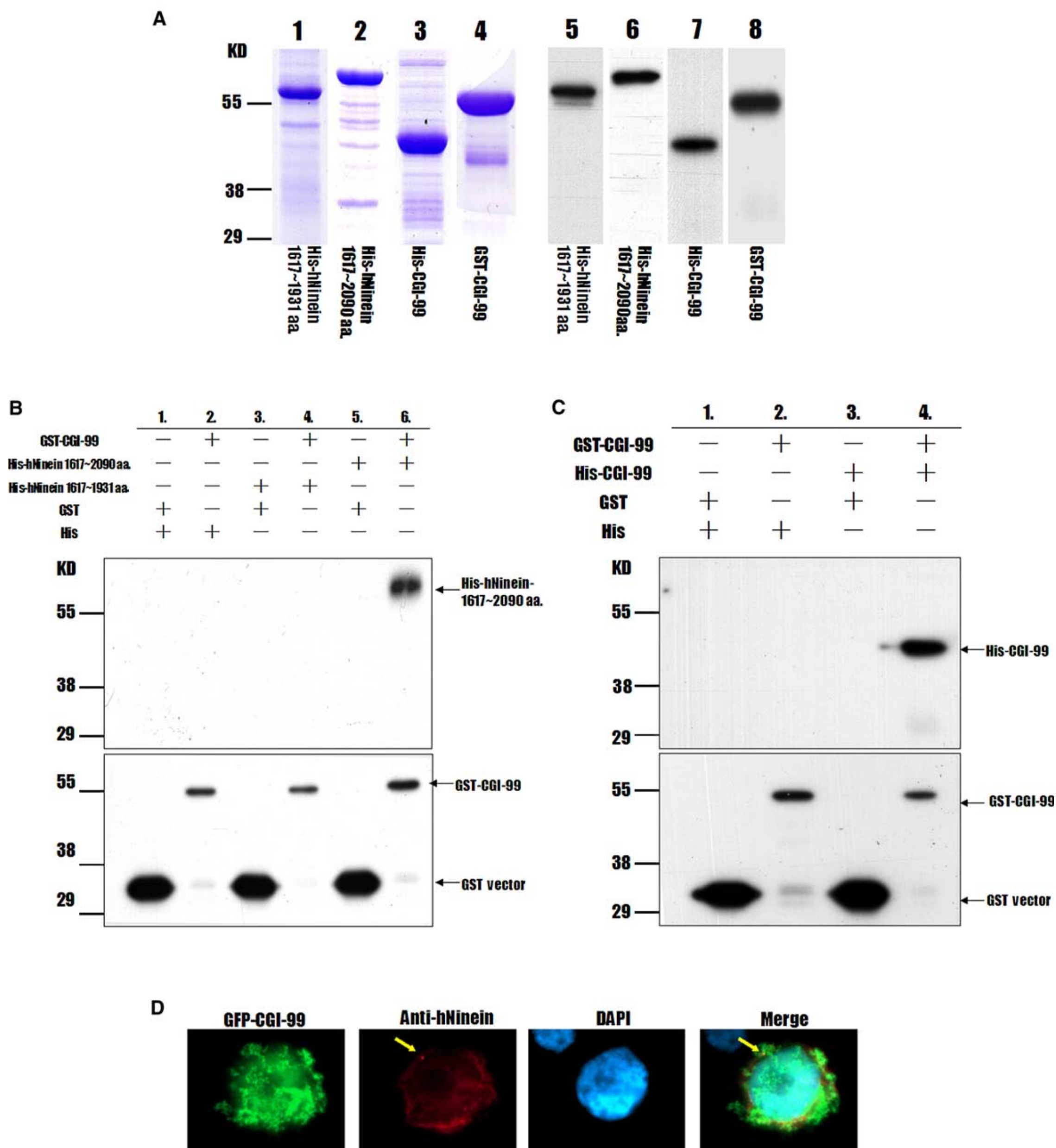


Fig. 4. In vitro GST-pull-down assays. (A) Coomassie blue staining (lanes 1–4); Western blotting with His antiserum (lanes 5–7); GST antiserum (lane 8). Lanes 1 and 5, His-tagged C-terminal hNinein (1617–1931 aa); lanes 2 and 6, His-tagged C-terminal hNinein (1617–2090 aa); lanes 3 and 7, His-tagged CGI-99; and lanes 4 and 8, GST-CGI-99. The molecular marker is shown on the left as indicated. (B) Interaction of CGI-99 and hNinein was analyzed either in the presence (+) or absence (−) of the assay mixtures containing purified GST-CGI-99; His-tagged C-terminal hNinein (1617–1931 aa); His-tagged C-terminal hNinein (1617–2090 aa); GST vector and His vector. The reactions were performed at 4 °C for 2 h and the reaction mixtures were finally analyzed by immunoblot analysis with anti-His antibody (upper panel); anti-GST antibody (lower panel). Arrow indicates positive interaction. (C) Dimerization of CGI-99 was analyzed either in the presence (+) or absence (−) of the assay mixtures containing purified GST-CGI-99; His-tagged-CGI-99; GST vector and His vector. The reactions were performed at 4 °C for 2 h and the reaction mixtures were finally analyzed by immunoblot analysis with anti-His antibody (upper panel); anti-GST antibody (lower panel). Arrow indicates positive interaction. (D) Co-localization of hNinein and CGI-99 as indicated by an arrow.

hNinein did not clearly show the centrosomal co-localization, they may co-localize at the spindle body during metaphase (Fig. 1C, lower panel). A recent paper using as proteomic approach of the human centrosome by protein correlation profiling [31] showed that hNinein and CGI-99 indeed co-existed in centrosome, implying that hNinein may interact with CGI-99. However, the biological function of CGI-99 and hNinein interaction remains to be elucidated.

3.4. CGI-99 interaction with hNinein blocks hNinein phosphorylation by GSK3β

Recently, several structurally distinct PKAs have been found to be localized at centrosomes and to regulate the function of centrosome [19–21]. It is also noted that hNinein, as we have identified previously, has been described as GSK3β interacting protein [22]. In our efforts, the kinase assay shows phosphorylation on 1617–2090 aa, but not on 1617–1931 aa fragment of

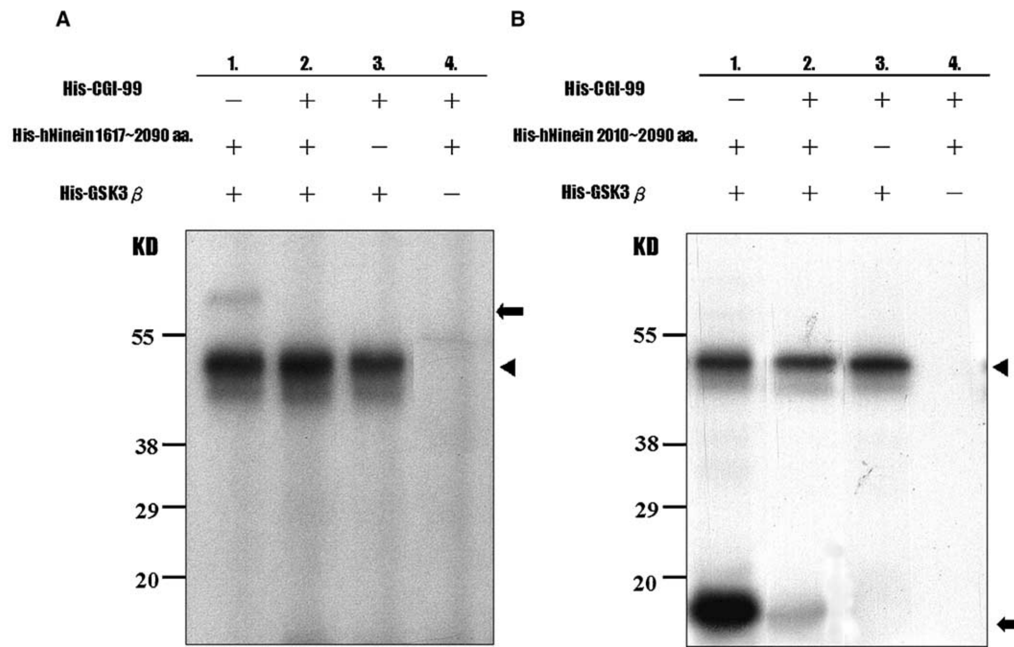
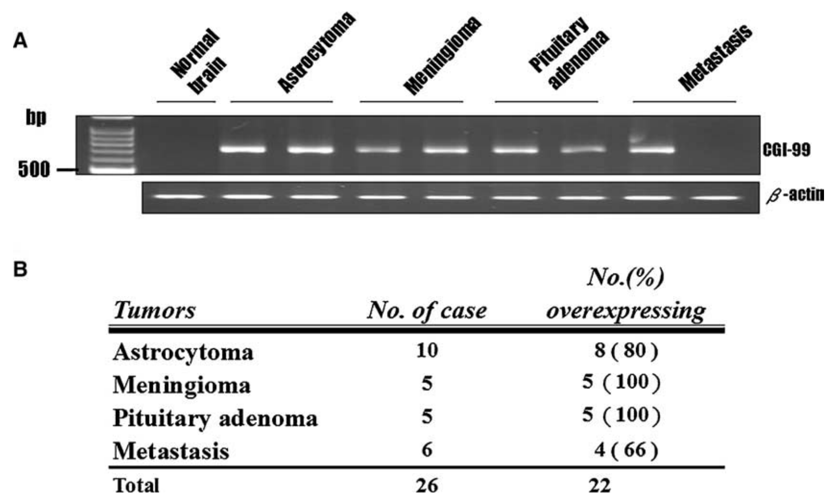


Fig. 5. Phosphorylation of the C-terminal fragment of hNinein by GSK3β. The reactions were analyzed either in the presence (+) or absence (−) of the assay mixtures containing purified His-tagged-CGI-99; His-tagged C-terminal hNinein (1617–2090 aa) or His-tagged C-terminal hNinein (2010–2090 aa) and partially purified His-tagged-GSK3β. (A) His-tagged C-terminal hNinein (1617–2090 aa). (B) His-tagged C-terminal hNinein (2010–2090 aa). Arrow indicates phosphorylated hNinein band. Arrowhead indicates GSK3β autophosphorylation.

hNinein, suggesting that hNinein phosphorylation site is located at more extensive C-terminal of hNinein (1617–2090 aa, Fig. 5A, lane 1). Indeed, the phosphorylation site of hNinein could be narrowed down to 80 amino acid residues at C-terminal of hNinein (2010–2090 aa), suggesting that 80 aa acts as a sufficient domain for GSK3β phosphorylation (Fig. 5B, lane 1). This result is in agreement with our previous observation showing that shorter C-terminal of hNinein fragment (1617–1931 aa) could not be phosphorylated by GSK3β [14]. In addition, CGI-99 does not act as a substrate for GSK3β (Figs. 5A and B, lane 3); however, our data show that CGI-99 interaction with hNinein could block hNinein phosphorylated by GSK3β (Figs. 5A and B, lane 2), suggesting that CGI-99 may be involved and restricted to hNinein phosphorylation during the cell cycle. It is noted that the region of hNinein binding to CGI-99 overlaps with GSK3β binding site (data not shown), suggesting that CGI-99 may be involved in the functional regulation of hNinein by competing with the hNinein phosphorylation site.

3.5. CGI-99 is highly expressed in brain tumors

To determine whether the data appearing in Northern blot or RT-PCR were true in Fig. 2, we further examined the CGI-99 expression profile in various brain tumors. In our studies, 26 human brain tumor samples were examined by RT-PCR. A representative experiment demonstrating amplification of CGI-99 from cancerous brain tissues is shown in Fig. 6A. Overall, 22 among 26 brain tumors exhibited CGI-99 overex-



Tumors	No. of case	No.(%) overexpressing
Astrocytoma	10	8 (80)
Meningioma	5	5 (100)
Pituitary adenoma	5	5 (100)
Metastasis	6	4 (66)
Total	26	22

Fig. 6. CGI-99 is expressed in brain tumors: (A) RT-PCR analysis of CGI-99 in various brain tumors. Data shown are representatives of at least three independent experiments. β-actin as internal control. (B) Distribution of CGI-99 in various brain tumors.

pression compared to normal brain tissue (Fig. 6B), suggesting that aberrant expression of the CGI-99 may contribute to the pathogenesis of brain tumors. It is noted that this study showed no significant difference in the CGI-99 overexpression between various brain tumors, even low-grade and high-grade brain tumors (data not shown); thus, these results suggest that malignant progression of brain tumors may not necessarily correlate with CGI-99 overexpression. It is likely that the overexpression of CGI-99 is involved in the early stage of brain tumor formation. It is also noted that CGI-99 transcript was indeed expressed in the fetal brain and lung more than in the adult brain and lung (Fig. 2B), implying that this protein may also be important in brain and lung development.

In conclusion, we have isolated a novel protein, CGI-99, which may be involved in the functional regulation of hNinein by interacting with hNinein and blocking hNinein phosphorylation, suggesting that CGI-99 could participate in the centrosome architecture as well as regulate centrosome formation. Moreover, its existence or overexpressions in fetal brain and brain tumors implies that this protein may also play a role in brain development and tumorigenesis as well.

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