Endogenous Human CaMKII Inhibitory Protein Suppresses Tumor Growth by Inducing Cell Cycle Arrest and Apoptosis through Down-regulation of the Phosphatidylinositol 3-Kinase/Akt/HDM2 Pathway

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Inhibition of calcium/calmodulin-dependent protein kinase II (CaMKII) results in hypophosphorylation of CaMKII substrates and in some cases suppresses cell growth. We previously presented the first report of the human CaMKII inhibitory protein, hCaMKIINβ. Here we report the functional characterization of hCaMKIINβ in ovarian cancer cells. We showed that hCaMKIINβ was highly expressed in normal ovarian tissues but was not detected in human ovarian adenocarcinoma, indicating that decreased expression of hCaMKIINβ may be involved in the pathogenesis of human ovarian adenocarcinoma. As an endogenous CaMKII inhibitor, hCaMKIINβ could significantly inhibit the growth of human ovarian cancer cells in vitro. In vivo, hCaMKIINβ decreased the tumorigenicity and growth of HO-8910PM human ovarian cancer cells and prolonged the survival of tumor-bearing mice. hCaMKIINβ blocked cell cycle progression and induced apoptosis of HO-8910PM cells, which was correlated with the up-regulation of p21, p53, and Bax and the down-regulation of cyclin A, cyclin D1, cyclin E, CDK2, phosphorylated retinoblastoma, and Bcl-2. We further demonstrated that hCaMKIINβ-mediated CaMKII inhibition suppressed Akt activation, leading to the down-regulation of HDM2, which was responsible for the up-regulation of p53 and p21 in human ovarian cancer cells. The tumor-suppressive effect and the negative expression in human ovarian cancer tissues suggest that hCaMKIINβ may play an important role in the regulation of tumor cell growth, possibly contributing to the development of new therapeutic strategies for ovarian cancer.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional calcium/calmodulin-dependent serine/threonine protein kinase. Recent studies suggest that CaMKII plays important roles in the control of cell cycle progression and cell proliferation (1–3). CaMKII inhibitors can inhibit CaMKII activity by interacting with the Ca2+/CaM-binding site or interfering with its catalytic activities. Previous studies of CaMKII inhibitors have been limited to chemically synthesized reagents such as KN-62 (4), KN-93 (5), and autocamtide-2-related inhibitory peptide, a synthetic inhibitory peptide (6), which have been shown to inhibit CaMKII-dependent processes in tumor and normal cells, causing cell cycle arrest, cellular apoptosis, or inhibition of cell proliferation (4, 5, 7, 8).

Up to now, four endogenous CaMKII inhibitory proteins (CaMKIINs) have been identified. Rat CaMKII inhibitory protein β (rCaMKIINβ) and α (rCaMKIINα), both identified from rat brain, were highly selective in inhibiting CaMKII activity, but their biological functions have not been elucidated (9, 10). hCaMKIINβ, an endogenous human protein that functions as an inhibitor of CaMKII, was first identified by our laboratory (11). We showed that overexpression of hCaMKIINβ could inhibit colon cancer cell growth in vitro, providing the first clue about the biological functions of the endogenous human CaMKII inhibitory protein (11). Recently, we have identified another human CaMKII inhibitory protein, hCaMKIINα, and demonstrated that it could inhibit human colon adenocarcinoma cell growth both in vitro and in vivo by arresting cell cycle at the S phase (12). Both hCaMKIINα and hCaMKIINβ have specific expression patterns in various tumor cells (11, 12); however, the signaling pathway involved in the control of tumor progression by these CaMKIINs, especially hCaMKIINβ, has not been specified.

Potential connections between Ca2+/CaMKII signaling and multiple signaling pathways have been reported in many cell types, among which the phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been implicated in a number of cell types in...
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response to a variety of stimuli, including growth factor withdrawal, cell cycle disturbances, loss of cell adhesion, and DNA damage (13–15). The tumor suppressor p53 is commonly inhibited under conditions in which the Akt pathway is activated (16). Intracellular levels of p53 are controlled by the E3 ubiquitin protein ligase, MDM2 (mouse double minute protein 2) (17, 18). The current model proposes that p53 and MDM2 form an autoregulatory feedback loop; p53 induces the transcription of MDM2, which in turn binds to the N-terminal transactivation domain of p53, thereby inactivating p53 transcriptional activity (17–19). A recent study has shown that Akt inhibits MDM2 self-ubiquitination via phosphorylation of MDM2 on Ser-166 and Ser-188 (20). Therefore, the activation status of the Akt pathway may be correlated with the expression and functions of p53 in tumor progression. However, the involvement of the Akt/MDM2 pathway in CaMKII signaling in the regulation of cell cycle progression has not been characterized yet.

Here we report the functional characterization of hCaMKIINβ in ovarian cancer cells. We showed that hCaMKIINβ was preferentially expressed in normal human ovarian tissues, but its expression was decreased in human ovarian cancer tissues. We also demonstrated that hCaMKIINβ could significantly inhibit the growth of human ovarian cancer cells via blocking cell cycle progression and inducing apoptosis. We further revealed that hCaMKIINβ up-regulated the expression of p53 and p21 through down-regulation of HDM2 expression by inactivating Akt. These findings suggest that hCaMKIINβ has potential antitumor effects on human ovarian cancer, thus providing a promising new strategy for the treatment of ovarian cancer.

EXPERIMENTAL PROCEDURES

Animals and Cell Lines—Five- to 6-week-old female athymic nu/nu mice (Sipper BK Experimental Animal Co., Shanghai, China) were housed in specific pathogen-free conditions. HO-8910PM, a highly metastatic human ovarian cancer cell line, was established by Zhejiang Cancer Hospital, China (21). Human cervix epithelioid carcinoma cells HeLa and human ovarian cancer cell lines CAOV-3 and SKOV-3 were obtained from the ATCC. Human ovarian cancer cell lines OVCAR-3, COC-1, and A-2780 were provided by China Center for Culture Collection (Wuhan University, Hubei, China).

Generation of Anti-CaMKIINβ Polyclonal Antibody—cDNA encoding full-length or the 41 N-terminal amino acids of hCaMKIINβ was cloned into pGEX-2T according to the instructions of the manufacturer (Amersham Biosciences). Soluble GST fusion proteins GST-KIINβ and GST-KIINβ-(1–41) were obtained under isopropyl 1-thio-β-d-galactopyranoside induction (0.2 mm) at 37 °C for 4 h and purified by glutathione-Sepharose 4B affinity chromatography per the manufacturer’s suggestions (Pierce). Polyclonal antibody to the recombinant GST-KIINβ protein (anti-hCaMKIINβ) was generated in rabbits against the fusion protein and purified using protein A affinity chromatography (Pierce).

Kinase Assay—CaMKII activity was assayed by incorporating [γ-32P]ATP (Amersham Biosciences) into autocamtide-2 (Calbiochem), a CaMKII-specific substrate peptide, using the CaMKII assay kit following the manufacturer’s instructions (New England Biolabs) (11). The amount of [γ-32P]ATP incorporated was determined using a liquid scintillation counter (Beckman Coulter).

Construction of Eukaryotic Expression Vector and Stable Transfection—Full-length coding region of hCaMKIINβ cDNA was inserted into pcDNA3.1/myc-His(−)B vector (Invitrogen) to generate the His-tagged expression vector, pKIINβ (11). CaMKIIα with His-282 mutated to Arg (H282R) was constructed by PCR cloning and PCR mutation. For stable transfection, HO-8910PM and HeLa cells were transfected with pKIINβ or empty vector (mock) using Lipofectamine, according to the manufacturer’s instructions (Invitrogen), and screened under 800 µg/ml G418 (Merck) for 4 weeks. Stably transfected clones were obtained by limiting dilutions and confirmed by Western blotting analysis using anti-hCaMKIINβ polyclonal antibody and were designated as HO-8910PM/pKIINβ, HO-8910PM/mock, HeLa/pKIINβ, or HeLa/mock, respectively.

Tissue Acquisition and RNA Preparation—Ovarian cancer tissues were obtained from surgical specimens from patients at the Zhejiang Cancer Hospital (Hangzhou, China). A total of 132 specimens from 81 pathologically confirmed epithelial ovarian carcinoma cases were used in this study. The age of the patients ranged from 33 to 65 years with a mean age of 53.5 years. Seventeen cases received pre- and post-operative chemotherapy, and 42 cases received post-operative chemotherapy. Chemotherapy regimen consisted of paclitaxel plus carboplatin (cisplatin) with a median of 4–6 cycles. Blocks of normal, margin, and malignant ovarian tissues were sharply dissected, and the total RNA was extracted with TRIzol reagent according to the manufacturer’s protocol (Invitrogen).

RT-PCR Analysis—Extraction of total cellular RNA from tissues and cells and first-strand cDNA synthesis were performed as described previously (12). PCR with the specific hCaMKIINβ primers 5′-GCCCTACAGGGAAGACAAGA-3′ (upstream) and 5′-TGCTCTATCCGGTCATCCT-3′ (downstream) were subjected to denaturation (95 °C, 30 s), annealing (57 °C, 45 s), and extension (72 °C, 30 s) for 32 cycles. The PCR products were confirmed by DNA sequencing.

Western Blotting, Co-precipitation, and Immunoprecipitation—Cells were lysed with lysis buffer (Cell Signaling Technology), and protein concentration was determined using the BCA protein assay (Pierce). Samples containing equal amounts of protein (50 µg) were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The blots were probed with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Reactive protein bands were visualized with enhanced chemiluminescence (Pierce) and viewed by gel documentation system UVP, c-80 (UVP Inc.). For co-precipitation, after transfection, whole cell lysates were extracted, and the His-tagged proteins were precipitated with nickel-nitritriacetate acid beads (Pierce). Alternatively, cell lysates were incubated with the relevant antibody followed by protein G-Plus-agarose (Santa Cruz Biotechnology). The immunoprecipitates were washed with cell lysis buffer and subjected to Western blot analysis.
Proliferation Assay In Vitro—The proliferation of tumor cells was measured by MTT dye reduction assay and [3H]thymidine incorporation as described previously (22, 23).

Colony Formation Assay—HO-8910PM/pKIINβ/H9252, HO-8910PM/mock, or parental HO-8910PM (500 cells/well) were seeded into 0.3% Bactoagar (Difco) over a 0.6% agar bottom layer in triplicate in 6-well plates. Both layers contained 1/1000 Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Plates were incubated at 37 °C in 5% CO2 for 3 weeks. Colonies greater than 100 mm in diameter were counted.

Cell Cycle Analysis—For flow cytometric analysis, 1 × 10⁶ cells were harvested and washed in PBS followed by fixation in 75% ice-cold ethanol for 30 min at 4 °C. After washing three times in cold PBS, the cells were resuspended in 1 ml of PBS and stained with propidium iodide at 37 °C for 30 min. Stained cells were then analyzed for their DNA content on a FACScalibur flow cytometer (BD Biosciences) (24).

Fluorescence Microscopy Analysis for Cellular Apoptosis—HO-8910PM/pKIINβ, HO-8910PM/mock, or parental HO-8910PM cells were plated in 24-well plates in RPMI 1640 medium supplemented with 2% fetal bovine serum and cultured for 24, 48, or 72 h. The cells were fixed with 4% paraformaldehyde and incubated with Hoechst 33342 (5 μg/ml, Sigma) for 15 min before examination under a fluorescence microscope. The extent of apoptosis was visualized and counted. Cellular apoptosis was also confirmed by transmission electron microscopy. Briefly, cells harvested as above were washed in PBS, and cell pellets were fixed in 4% paraformaldehyde. The cell pellets were then loaded onto electron microscopy grids coated with Formvar carbon, and subsequently contrasted and embedded in a mixture of uranyl acetate and methylcellulose. Sections were observed by a Philips Tecnai-10 transmission electron microscope operating at 80 kV (Phillips Electronic Instruments, Rahway, NJ) (magnification, ×3700).
RNA Interference—The hCaMKIINβ-specific siRNA (si-KIINβ, 5'-CUCUUUGUUACUGGCUCUUTT), HDM2-specific siRNA (5'-GACUAUCUCAGCCAUCAA), and scrambled control (si-Non, 5'-UUCUGGACAGGUCACGCUTT) were synthesized by GeneChem (Shanghai, China). siRNA was delivered into the cells using Oligofectamine (Invitrogen).

Tumorigenicity of HO-8910PM Cells Overexpressing hCaMKIINβ—HO-8910PM/pKIINβ, HO-8910PM/mock, or parental HO-8910PM cells (1.0 × 10⁶ each) were subcutaneously injected into female nude mice. Tumor growth was measured using caliper every 3 days, and the tumor volumes were calculated according to the following formula: volume = length × (width²)/2.

Antitumor Effects of hCaMKIINβ in Vivo—HO-8910PM cells (1.0 × 10⁶) were subcutaneously injected into the female nude mice. When the tumors sizes were ~3–4 mm in diameter, mice were randomly divided into three groups. Each group contained eight mice. 20 μg of hCaMKIINβ plasmid DNA, control vector, and PBS (all in 30 μl volume) were injected into the tumors of HO-8910PM-bearing mice by electroporation as described previously (26). The site in which the plasmid was injected was sandwiched in an electrode (BTX 533 two-needle array electrode) with poles 5 mm in diameter. Three electrical pulses of 20-ms duration at a voltage of 600 V were delivered using an ECM 830 Electro Square porator (BTX, San Diego). Tumor growth was monitored with a caliper every 3 days according to the following formula: volume = length × (width²)/2. Survival of HO-8910PM-bearing mice was recorded daily.

Statistical Analysis—Statistical analysis was performed using Student’s t test, and long rank test was used for survival analysis. The statistical significance was determined at p < 0.05.

RESULTS

Expression of hCaMKIINβ, an Endogenous CaMKII Inhibitory Protein, Was Negatively Correlated with the Progression of Human Ovarian Adenocarcinoma—We first determined whether hCaMKIINβ was an endogenous CaMKII inhibitor. First, immunoprecipitation assay revealed that endogenous hCaMKIINβ interacted with CaMKII in HeLa cells (positive for hCaMKIINβ expression) (Fig. 1A). Then in vitro kinase assay based on substrate phosphorylation by CaMKII showed that CaMKII kinase activity was significantly suppressed by the recombinant hCaMKIINβ protein, GST-KIINβ, in a dose-dependent manner (Fig. 1B). However, GST-KIINβ (1–41) without the C-terminal 27-residue conserved inhibitory region (27CIR) did not exhibit any inhibitory effect. Autonomous CaMKII activity was significantly inhibited by hCaMKIINβ overexpression in HO-8910PM ovarian cancer cells, including HO-8910PM, CAOV-3, SKOV-3, and A-2780 cells (negative for endogenous hCaMKIINβ expression, see Fig. 2A), but increased by down-regulation of hCaMKIINβ expression in HeLa cells by using hCaMKIINβ-specific siRNA (si-KIINβ) (Fig. 1, C and D). Taken together, these results demonstrated that hCaMKIINβ was an endogenous CaMKII inhibitor that could effectively interact with CaMKII and inhibit its kinase activity.

hCaMKIINβ was found to inhibit the cell proliferation of human colon adenocarcinoma LoVo cells (11), which was consistent with the observations that inhibition of calmodulin kinase might be involved in the control of tumor cell growth (27, 28). However, there has been no report about the role of the CaMKII inhibitory protein in the control of ovarian cancer progression. So hCaMKIINβ expression in several ovarian cancer cells was first examined here. We found that hCaMKIINβ mRNA was not expressed in ovarian cancer cells, including HO-8910PM, CAOV-3, SKOV-3, A-2780, OVCAR-3, and COC-1 cells (Fig. 2A). The expression pattern was further confirmed by Western blotting analysis with anti-hCaMKIINβ polyclonal antibody (Fig. 2A). We then evaluated hCaMKIINβ expression in clinical specimens of normal ovarian tissues and ovarian cancer tissues from Chinese females. As shown in Fig. 2B, there was no detectable expression of hCaMKIINβ in the two samples of human ovarian cancer tissue, as determined by RT-PCR (lane 1 and lane 2 represent two different samples). However, benign lesions (Fig. 2B, lanes 3 and 4) and normal ovarian tissue samples displayed strong hCaMKIINβ expression (Fig. 2B, lanes 5 and 6 represent two different samples). An
additional 132 ovarian tissue samples, including 81 cases of ovarian adenocarcinomas, 19 cases of tumor margin tissues, and 32 cases of normal ovarian tissues, were further tested for the expression of hCaMKIINβ mRNA by RT-PCR analysis. The expression of hCaMKIINβ was shown to be present in a very high percentage of normal ovarian tissue (90.6%, 29:32) and of margin tissue (89.5%, 17:19) but in a relatively low percentage of ovarian cancer tissue samples (38.3%, 31:81; \( p < 0.0001 \) versus normal and margin group, see Fig. 2C). These results indicated a negative hCaMKIINβ expression in human ovarian cancer, suggesting that hCaMKIINβ may be involved in the pathogenesis of ovarian adenocarcinoma.

hCaMKIINβ Overexpression Inhibited the Proliferation and Colony Formation of Human Ovarian Cancer Cells—To investigate the biological functions of hCaMKIINβ in ovarian cancer cells, hCaMKIINβ-negative HO-8910PM human ovarian cancer cells were transfected with hCaMKIINβ expression vector (pKIINβ) or mock vector (supplemental Fig. S1A). We first observed the morphological change of HO-8910PM cells overexpressing hCaMKIINβ by phase contrast microscopy. 24–72 h post-transfection, parental HO-8910PM cells and mock cells grew as a tightly adherent monolayer, whereas HO-8910PM/hCaMKIINβ cells became detached and exhibited a rounded shape, and then became smaller and contracted (Fig. 3A). However, hCaMKIINβ did not alter the morphology of HeLa cells (data not shown), which was consistent with our previous observation (11).

We then evaluated the effect of hCaMKIINβ overexpression on the proliferation of human ovarian cancer cells in vitro by MTT assay. Cells were transfected with hCaMKIINβ expression vector (pKIINβ) or mock vector, and the expression of hCaMKIINβ was confirmed by Western blot using anti-hCaMKIINβ antibody (Fig. 3B). After 24–48 h of cell plating, the proliferation of stably transfected HO-8910PM cells was obviously inhibited. After 72 h, the cell proliferation was inhibited 3-fold in hCaMKIINβ-overexpressing cells over that in parental or mock cells (Fig. 3B, \( p < 0.01 \)). However, hCaMKIINβ had no effect on the growth of HeLa cells (Fig. 3C), which was also consistent with our previous report (11). \(^{[3H]}\)Thymidine incorporation assay also confirmed the inhibitory effect of hCaMKIINβ overexpression on the growth of HO-8910PM cells in vitro (Fig. 3D, \( p < 0.01 \)). A soft agar colony formation assay was further per-
formed to assay the effect of hCaMKIIN/β overexpression on the anchorage-dependent growth of HO-8910PM cells. As shown in Fig. 3E, the number of colonies derived from HO-8910PM/pKIIN/β was markedly reduced compared with that from HO-8910PM/mock and parental HO-8910PM cells. To further confirm the role of hCaMKIIN/β, we examined the effects of knockdown of endogenous hCaMKIIN/β by hCaMKIIN/β-specific siRNA (si-KIIN/β) in HeLa cells. Down-regulation of hCaMKIIN/β expression markedly accelerated the growth of HeLa cells (Fig. 3F), suggesting that not only exogenous hCaMKIIN/β but also endogenous hCaMKIIN/β could inhibit tumor growth. Besides, hCaMKIIN/β lost its tumor-suppressing ability on HO-8910PM cells when cells were pretreated by KN-62, a specific chemical inhibitor for CaMKII (supplemental Fig. S2), suggesting that the inhibitory effect of hCaMKIIN/β required its inhibitory capacity to CaMKII activity. Together, these results suggested that overexpression of hCaMKIIN/β significantly inhibited the growth of human ovarian cancer cells through inhibition of CAMKII activity in vivo.

**hCaMKIIN/β Inhibited the Growth of HO-8910PM Cells in Vivo**—As illustrated in Fig. 4A, we showed that tumorigenicity of HO-8910PM/pKIIN/β cells was significantly decreased as compared with that of parental or mock cells ($p < 0.01$). We further investigated whether hCaMKIIN/β overexpression in human ovarian adenocarcinoma could mediate therapeutic effects in vivo. HO-8910PM tumor-bearing nude mice were used as a model and the purified hCaMKIIN/β expression plasmid DNA (pKIIN/β) was delivered into the tumors by in vivo electroporation. The results showed that intratumoral gene transfer of hCaMKIIN/β significantly inhibited the growth of HO-8910PM tumors as compared with mock DNA (Fig. 4B, $p < 0.01$). Accordingly, the survival of HO-8910PM-bearing nude mice was prolonged most significantly by intratumoral gene transfer of hCaMKIIN/β, with 50% of the mice surviving for 90 days, whereas the HO-8910PM-bearing nude mice of both mock DNA group and PBS group died within 65 days (Fig. 4C, $p < 0.01$). Therefore, intratumoral gene delivery of human hCaMKIIN/β can significantly suppress in vivo growth of ovarian cancer.

**hCaMKIIN/β Overexpression Induced Cell Cycle Arrest and Apoptosis of Human Ovarian Cancer Cells**—To investigate the underlying mechanism(s) whereby hCaMKIIN/β inhibited ovarian cancer cell growth, we determined the cell cycle distribution of HO-8910PM/pKIIN/β cells by fluorescence-activated cell sorter analysis 24, 48, and 72 h after transfection. As shown in Fig. 5A, a higher proportion of HO-8910PM/pKIIN/β cells accumulated in the G$_0$/G$_1$ phase (73.06%) than in the parental (54.70%) or mock cells (53.43%). There was a concurrent decrease in the proportion of HO-8910PM/pKIIN/β cells at the G$_2$–M phase. The results suggested that a cell cycle checkpoint was activated to block the entry of these cells into the S phase. Moreover, the accumulation of cells in the S phase caused by hCaMKIIN/β overexpression pointed to the apparent activation of another checkpoint, one at the exit from S phase. Therefore, the data suggested the activation of (at least) two checkpoints by hCaMKIIN/β to regulate the cell cycle progression in human ovarian cancer cells.

After transient transfection of hCaMKIIN/β, HO-8910PM cells displayed shrinkage and loss of adherence and became rounded (data not shown), suggesting that hCaMKIIN/β overexpression might induce apoptosis. HO-8910PM/pKIIN/β, HO-8910PM/mock, and HO-8910PM cells were cultured in RPMI 1640 medium containing 2% fetal bovine serum for 24, 48, and 72 h, respectively. The cell populations were then stained with Annexin V-FITC and PI (propidium iodide) and quantified by flow cytometry. As shown in Fig. 5B, a higher proportion of HO-8910PM/pKIIN/β cells showed an increased rate of apoptosis as compared with parental or mock cells ($p < 0.01$). The results suggested that hCaMKIIN/β overexpression might induce apoptosis.
These results demonstrated that induction of apoptosis could be one of the mechanisms whereby hCaMKIINβ inhibited the proliferation of HO-8910PM cells.

hCaMKIINβ Overexpression Up-regulated p21, p53, and Bax but Down-regulated Cyclin A, Cyclin D1, Cyclin E, CDK2, Bcl-2, and Phosphorylated Retinoblastoma in Human Ovarian Cancer Cells—Cell cycle and apoptosis involve complex molecular cascades; dysfunction of a variety of proteins may lead to the blockage of cell cycle progression and the induction of apoptosis. To further explore the molecular mechanisms for hCaMKIINβ-induced cell cycle arrest in the G0/G1 phase, we tested the expression levels of several cell cycle regulators in HO-8910PM cells following transient transfection of hCaMKIINβ. Western blot analysis showed that expression of cyclin A, cyclin D1, cyclin E, CDK2, and the phosphorylation level of retinoblastoma in HO-8910PM/pKiINβ cells was markedly decreased at 24, 48, and 72 h post-transfection (Fig. 6A). However, the level of cyclin B1 remained unchanged. The tumor suppressor genes p21 and p53 have been shown to regulate the cell cycle progression through different mechanisms. p21 binds to and universally inhibits the cyclins/CDKs, thus inhibiting cell cycle progression, and p53 regulates the cell cycle at the G1 checkpoint. We found that the expression of p21 and p53 was greatly increased in HO-8910PM/pKiINβ cells but not in parental or mock cells (Fig. 6A and supplemental Fig. S4A). These results suggested that hCaMKIINβ may down-regulate the expression of critical cell cycle regulators, thereby leading to cell cycle arrest of HO-8910PM cells. Bcl-2 family proteins play key roles in cellular apoptosis. As shown in Fig. 6A, transient expression of hCaMKIINβ could markedly increase the expression of the apoptosis-related protein Bax but decrease the expression of the anti-apoptotic protein Bcl-2 in HO-8910PM cells. These findings suggested that up-regulation of Bax and down-regulation of Bcl-2 in HO-8910PM cells may be involved in hCaMKIINβ-induced apoptosis.

We also analyzed the mRNA expression of these cell cycle and apoptosis regulators in three matched normal ovarian and cancer tissue samples freshly isolated from the ovarian cancer patients. We found that hCaMKIINβ mRNA was undetectable in all three cancer tissue samples but expressed at different levels in matched normal tissue samples. Our preliminary data using human cDNA plate arrays (Signosis) confirmed the up-regulation of p53, p21, and down-regulation of HDM2 mRNA in the normal ovarian tissues compared with that in freshly isolated ovarian cancer tissues (data not shown).

Inhibition of PI3K/Akt/HDM2 Pathway Was Responsible for the Up-regulation of p53 by hCaMKIINβ-mediated CaMKII Inhibition in Human Ovarian Cancer Cells—Among those that were regulated by hCaMKIINβ, p53 may stand in a central position, because the growth of OVCAR-3 cells (hCaMKIINβ-negative, p53 mutant) was barely affected by hCaMKIINβ overexpression (supplemental Fig. S5). We then proceeded to explore the signal pathway(s) that contributed to the up-regulation of p53 by hCaMKIINβ. Activation of the PI3K/Akt pathway is one of the critical steps in cell survival through anti-apoptosis (29). A previous study demonstrated that CaMKII can activate the PI3K/Akt pathway (30, 31). To investigate whether the PI3K/Akt pathway was regulated by hCaMKIINβ, we measured...
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It has been shown that inhibition of Akt activity impairs the phosphorylation of the HDM2 (human homologue of MDM2), resulting in the destabilization of HDM2. This prompted us to investigate whether inhibition of Akt by hCaMKIINβ affected HDM2 phosphorylation and protein levels in hCaMKIINβ-transfected HO-8910PM cells. We found that hCaMKIINβ was able to reduce the phosphorylation and level of HDM2 in transfected HO-8910PM cells (Fig. 6B). In H282R-transfected HO-8910PM cells, increased Akt phosphorylation and HDM2 expression were observed, which was consistent with the results of hCaMKIINβ-mediated inactivation of Akt and HDM2 down-regulation. Besides LY294002 treatment almost completely abrogated the up-regulation of HDM2 in HO-8910PM cells transfected with H282R (Fig. 6C), suggesting that the down-regulation of HDM2 by hCaMKIINβ was dependent on the inhibition of the PI3K/Akt signaling pathway.

Then what is the role of HDM2 in the hCaMKIINβ-mediated antitumor effect? HO8910-PM cells were transfected with HDM2-specific siRNA (si-HDM2) or si-Non for 24 h, and then HO8910-PM cells were transfected with H282R for 48 h, and the expression of p53 and p21 was detected by Western blotting. The expression of p53 and p21 was hardly affected by hCaMKIINβ overexpression in HDM2 siRNA (si-HDM2)-transfected HO8910-PM cells (Fig. 6D), suggesting that HDM2 played an important role in hCaMKIINβ-mediated up-regulation of p53 and p21.

Taken together, these results indicate that the de-activation of Akt signaling by hCaMKIINβ-mediated CaMKII inhibition may be responsible for the up-regulation of p53 and p21 expression through decreasing HDM2.

DISCUSSION

Chemical CaMKII inhibitors such as KN-62, KN-93, and autocamtide 2-related inhibitory peptide have been found to strongly inhibit CaMKII activity and functions. To date, CaMKII inhibition has been found to induce cell cycle arrest or cellular apoptosis in a variety of malignant or normal cells (4, 5, 7). G1 and/or S phase arrest of K562, NIH 3T3, and HeLa cells was induced by KN-62 or KN-93 treatment (4, 5, 7). G1 phase arrest is also induced in human and mouse fibroblasts by calcium deprivation (32). We have previously shown that the human CaMKII inhibitory protein, hCaMKIINβ, which was first identified in our laboratory, could inhibit the proliferation of LoVo cells in vitro (11). hCaMKIINα, another human CaMKII inhibitory protein identified by us, inhibits human colon adenocarcinoma cell growth both in vitro and in vivo by arresting cell cycle at the S phase (12). In this study, we describe the functional characterization of hCaMKIINβ, whose expression is negatively correlated with the development of human ovarian adenocarcinoma. Here we provide strong evidence for a significant inhibitory effect of hCaMKIINβ on the growth of ovarian cancer cells in vitro and in vivo, and we show that these inhibitory effects are associated with the induction of cell cycle arrest and apoptosis. We demonstrate that hCaMKIINβ-medi-
ated up-regulation of p21, p53, and Bax and down-regulation of cyclin A, cyclin D1, cyclin E, CDK2, phosphorylated retinoblastoma, and Bcl-2 may contribute to the molecular mechanisms for hCaMKII\(\beta\) to induce cell cycle arrest and apoptosis. In addition, we found that hCaMKII\(\beta\) up-regulates the expression of p53 and p21 through down-regulating the expression of HDM2 by inactivating the PI3K/Akt pathway.

The outstanding features of hCaMKII\(\beta\)-expressing cells are the cell cycle arrest at the G\(_0\)/G\(_1\) phase and decreased expression of cyclin A, cyclin E, CDK2, and the accumulation of p21 and hypophosphorylated Rb. We suggest that these events might be linked and that CDK2, p21, and Rb play key roles in the cellular response to hCaMKII\(\beta\). Cyclin-CDK complex plays an important role in regulating cell cycle progression. Cyclin E-CDK2 activity is required for progression from G\(_1\) to S phases; cyclin A-CDK2 activity is required for the S progression. Regulation of cyclin-CDK complexes has been shown at several levels. These include transcriptional regulation, stabilization of protein, increased phosphorylation of CDK through cyclin-dependent kinase-activating kinases, or inhibition of the cyclin-dependent phosphatases and association with cyclin-dependent kinase inhibitors. In this study, although not tested, the up-regulation of p21 and the deregulation of cyclin E, cyclin A, and CDK2 mediated by hCaMKII\(\beta\) in HO-8910PM cells might contribute to the down-regulation of cyclin E-CDK2 and cyclin A-CDK2 complexes. On the other hand, because Rb has the potential to interfere with G\(_1\)-S phase progression (33, 34), the reduced levels of cyclin E, cyclin A, and CDK2, which are all E2F target genes (35), can be attributed to the accumulation of active Rb. So the modulation of different cell cycle regulatory proteins might be a consequence of deactivation of CDK2 and activation of Rb, which we observed. Different mechanisms can be envisaged for the activation of Rb; either Rb kinases could be inhibited or a Rb phosphatase could be induced. We observed that the expression of cyclin E, cyclin A, and CDK2 was reduced, which raised the possibility that hCaMKII\(\beta\)-mediated appearance of hypophosphorylated Rb could be achieved by the Rb kinases, especially by cyclin A and/or cyclin E-associated CDK2. However, we cannot exclude the possibility that an Rb phosphatase was also induced, although little is known about how the phosphatases are induced following different stimuli. In conclusion, our findings show that cyclin A, cyclin E, CDK2, p21, and Rb are major inter-players in the hCaMKII\(\beta\)-mediated G\(_0\)/G\(_1\) arrest.

PI3K is involved in multiple cellular functions, including proliferation, differentiation, anti-apoptosis, tumorigenesis, and angiogenesis (29, 36). Recent studies demonstrate that the gene encoding the p110\(_\alpha\) catalytic subunit of PI3K is frequently amplified in ovarian cancer cells. PI3K inhibitor LY-294002 has been shown to inhibit ovarian cancer cell proliferation by inducing G\(_1\) cell cycle arrest (37). Inactivation of PI3K activity markedly inhibits the proliferation of small cell lung cancer cells by promoting cell cycle delay in G\(_1\) (38). Therefore, PI3K and Akt activities are important for the G\(_1\) cell cycle progression in ovarian cancer cells and other tumor cells. Our findings that hCaMKII\(\beta\) down-regulates the PI3K/Akt pathway, and at the same time arrests cell cycle at G\(_0\)/G\(_1\) in HO-8910PM cells, imply that the PI3K/Akt pathway may be involved in hCaMKII\(\beta\)-induced G\(_1\) cell cycle arrest. We went further to address how the PI3K/Akt pathway mediates the modulation of cell cycle progression in human ovarian cancer cells overexpressing hCaMKII\(\beta\). We have shown that hCaMKII\(\beta\) is capable of up-regulating p53 expression and down-regulating HDM2 expression. It has been reported that the stoichiometric balance between p53 and its negative regulator, MDM2/MDM3. 

![FIGURE 7. A model depicting the mechanisms for the induction of human ovarian cancer cell cycle arrest and apoptosis by hCaMKII\(\beta\)-mediated inhibition of CaMKII.](image-url)
Akt/HDM2 pathway that hCaMKIIN Ser-188. Therefore, it seems through inactivation of the PI3K/ubiquitination via phosphorylation of MDM2 on Ser-166 and in fact, a recent study showed that PI3K inhibits MDM2 self-approach for treatment of ovarian cancer. Our results suggest that hCaMKII inhibitory protein mediates CaMKII in inhibiting the expression of HDM2, may determine the extent of cellular p53 activity and its functions. MDM2 harbors a self- and p53-specific the E ubiquitin protein ligase activity within its evolutionarily conserved C-terminal RING finger domain, which mediates p53 ubiquitination and rapid degradation by the 26 S proteasome (39). The current model holds that p53 and MDM2 form an autoregulatory feedback loop; MDM2 transcription is induced by p53, and MDM2 in turn binds to the N-terminal transactivation domain of p53, thereby inactivating p53 transcriptional activity (17, 19). Antitumor Effect of CaMKII Inhibitory Protein

One plausible explanation is that the inactivation of PI3K/Akt/HDM2-mediated CaMKII inhibition may up-regulate p53 expression, resulting in cell cycle arrest and cellular apoptosis (Fig. 7).

Ovarian cancer accounts for 4% of all cancers in women and has the highest mortality among the gynecologic malignancies (40). The current therapeutic approach is not very effective in ovarian cancer patients. Better understanding of molecular mechanisms for the induction of cell cycle arrest and cellular apoptosis will contribute to the design of effective and rational therapy of ovarian cancer. Our results suggest that hCaMKIINβ induces cell cycle arrest and cellular apoptosis of ovarian cancer cells, resulting in significant inhibition of ovarian cancer growth both in vitro and in vivo. These results indicate that gene transfer of hCaMKIINβ may be a new therapeutic approach for treatment of ovarian cancer.

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