A Novel Endogenous Human CaMKII Inhibitory Protein Suppresses Tumor Growth by Inducing Cell Cycle Arrest via p27 Stabilization*\$\n
Chunmei Wang 1, Nan Li 1, Xingguang Liu, Yuanyuan Zheng, and Xuetao Cao 2

From the Institute of Immunology and National Key Laboratory of Medical Immunology, Second Military Medical University, 800 Xiangyin Road, Shanghai 2010433, China

Calcium/calmodulin-dependent protein kinase II (CaMKII) regulates numerous physiological functions. Inhibition of CaMKII activity, mostly by synthetic reagents, has been proved to suppress cell growth in many cases. So far there are no reports about the physiological functions and underlying mechanisms of endogenous CaMKII inhibitory proteins in cell cycle progression. Here we report the characterization of a novel human endogenous CaMKII inhibitor, human CaMKII inhibitory protein α (hCaMKIINα), which directly interacts with activated CaMKII and effectively inhibits CaMKII activity. hCaMKIINα expression is negatively correlated with the severity of human colon adenocarcinoma. Overexpression of hCaMKIINα inhibits colon adenocarcinoma growth in vitro and in vivo by arresting the cell cycle at the S phase through its conserved inhibitory region (27CIR), whereas silencing the hCaMKIINα expression accelerates tumor growth and cell cycle progression. We found that the effect of hCaMKIINα on cell cycle is correlated with up-regulation of p27 expression, which may be due to the inhibition of proteasome degradation, but not transcriptional regulation, of p27. Moreover, hCaMKIINα deactivated MEK/ERK, which is prerequisite to the inhibition of Thr-187 phosphorylation and subsequent proteasomal degradation of p27, causing the inhibition of S-phase progression of cell cycle. The findings underscore a link between hCaMKIINα-mediated inhibition of CaMKII activity and p27-dependent pathways in controlling tumor cell growth and cell cycle and imply a potential application of hCaMKIINα in the therapeutics of colon cancers.

Calcium (Ca\(^{2+}\)) is a universal second messenger that regulates a broad range of cellular processes, including cell development, proliferation, motility, secretion, and others (1, 2). Members of the Ca\(^{2+}/\text{calmodulin (CaM)}\)-dependent protein kinase (CaMK) family are biochemical decoders of intracellular Ca\(^{2+}\) oscillations (3, 4), among which CaMKII is a ubiquitous serine/threonine protein kinase that phosphorylates nearly 40 different proteins, including enzymes, ion channels, kinases, and transcription factors (5, 6). Therefore, CaMKII is critical for many physiological and pathological functions of cells, and how to regulate CaMKII activity is an important question in the field of biomedicine.

CaMKII inhibitors can block CaMKII activity by connecting Ca\(^{2+}/\text{CaM binding site or affecting its catalytic function. The CaMKII inhibitors used in the previous studies were the synthesized chemical reagents such as KN-62 (7) and KN-93 (8), or synthetic inhibitory peptide such as AIP (9). These inhibitors of CaMKII have been shown to inhibit CaMKII-dependent function in tumor cells, causing cell growth inhibition by impairment of cell cycle progression or induction of apoptosis (10–13). The effect of CaMKII inhibitors on cell cycle was associated with changed expression levels of cell cycle-related proteins (6, 11). For example, treatment of HeLa cells with KN-93 results in a cell cycle blockade in the G2 phase. Similarly, KN-93 could decrease cyclin-dependent kinase (cdk) 4 activity by reducing cyclin D1 levels and cdk2 activity by enhancing p27\(^{kip1}\) (p27) expression, causing cell cycle arrest at the G2 phase. Up to now, three endogenous CaMKII inhibitory proteins have been identified. Two rat brain-derived CaMKII inhibitors rCaMKINβ and rCaMKINα interact with the activated CaMKII and inhibit CaMKII activity (14, 15). We have identified a human CaMKII inhibitory protein, hCaMKIINβ, and shown that it inhibits human colon adenocarcinoma cell growth (16). However, Up to now there is no report about the physiological functions and the underlying mechanisms of endogenous CaMKII inhibitors in cell cycle progression.

On the basis of identification of hCaMKIINβ (16), here we report the functional characterization of another novel endogenous human CaMKII inhibitory protein, designated as human CaMKII inhibitory protein α (hCaMKIINα), and hypothesize...

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Inst. of Immunology and National Key Laboratory of Medical Immunology, Second Military Medical University, Shanghai 2010433, P.R. China. Tel.: 86 21 55620605; Fax: 86 21 6538 2502; E-mail: caoxt@public3.sta.net.cn.

3 The abbreviations used are: CaM, calmodulin; CaMKII, calcium/calmodulin-dependent protein kinase II; cdk, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; hCaMKIIN, human calcium/calmodulin-dependent protein kinase II inhibitor alpha; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; siRNA, small interference RNA; GST, glutathione S-transferase; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorting; JNK, c-Jun N-terminal kinase.
that hCaMKII\(\alpha\) has suppressor effects on colon tumorigenesis. We find a negative correlation of hCaMKII\(\alpha\) expression with the severity of human colon adenocarcinoma, and hCaMKII\(\alpha\) can suppress growth of colon adenocarcinoma both in vitro and in vivo. We also demonstrate that the effect of hCaMKII\(\alpha\) on cell cycle is correlated with up-regulation of p27 expression, which may be due to the inhibition of proteasome degradation but not transcriptional regulation of p27. Moreover, hCaMKII\(\alpha\) deactivated MEK/ERK, which is prerequisite to the inhibition of Thr-187 phosphorylation and subsequent proteasomal degradation of p27, causing the inhibition of S-phase progression of cell cycle. The findings suggest that the novel endogenous human CaMKII inhibitor hCaMKII\(\alpha\) may be a functional growth inhibitor for colon tumor cells by inducing p27 accumulation and then cell cycle arrest, and represents a potential manner to control colon cancer development.

**EXPERIMENTAL PROCEDURES**

**Cells, Reagents, and Antibodies**—Human colon adenocarcinoma cells, including LoVo, HT-29, LS-174T, SW480, and SW620, were all obtained from ATCC. MG132 and U0126 were from Calbiochem, and cycloheximide (cycloheximide) and ionomycin were from Sigma. Anti-His, p27, phosphorylated p27(T187), and MEK1 were from Santa Cruz Biotechnology, Santa Cruz, CA; anti-ERK1/2, phosphorylated MEK1, and phosphorylated ERK1/2 antibodies were from Cell Signaling Technology; and anti-FLAG, actin, and CaMKII\(\alpha\) antibodies were from Sigma. An hCaMKII\(\alpha\)-specific polyclonal antibody was raised against affinity chromatography-purified GST-KIIN\(1–41\) fusion protein, and the antibody specificity was confirmed by Western blot and immunohistochemical staining for no cross-reactivity with hCaMKIIN\(\beta\).

**Molecular Cloning and Vector Construction**—The full-length hCaMKII\(\alpha\) cDNA was assembled in silico by searching the NCBI data base and amplified by reverse transcription-PCR from bone marrow stromal cells. The His-tagged expression vectors of full-length and domain-truncated mutants of hCaMKII\(\alpha\) (as illustrated in Fig. 1C), including pKIIN\(\alpha\), pKIIN\(\alpha\(_1–41\), pKIIN\(\alpha\(_1–53\), pKIIN\(\alpha\(_1–68\), and the FLAG-tagged expression vectors of CaMKII\(\alpha\) (pFLAG-CaMKII), and CaMKII\(\alpha\) with H282 mutated to R (H282R) were constructed by PCR cloning and PCR mutation. The hCaMKII\(\alpha\) siRNA-generating plasmid, pl-kiIN\(\alpha\), was constructed using the GeneSuppressor system (Imagenex). Vectors were transfected into cells using Lipofectamine2000 reagent (Invitrogen) according to the manufacturer’s instructions. Unless specified, cells were subjected to analysis 48 h post-transfection. To express GST fusion proteins, the code region of hCaMKII\(\alpha\) and its mutants were cloned in-frame into pGEX-4T3 vector (Amersham Biosciences).

**Tissue Microarray**—The human tissue arrays of normal and neoplastic colon tissues (Cybrdi, Shanxi, China) were fixed with formalin, embedded with paraffin, and immunostained with anti-hCaMKII\(\alpha\) antibody by standard immunohistochemistry protocol as described previously (17). The secondary antibody was used alone as a negative control.

**RNA Interference**—The p27 siRNA was purchased from Santa Cruz Biotechnology. The hCaMKII\(\alpha\)-specific siRNA (si-KIIN\(\alpha\), 5’-GCAAGCGGGUUGUAAUGATT) and scrambled control (si-Non, 5’-UUCUCCGAACGUGUCAGUTT) were synthesized by GeneChem (Shanghai, China). siRNA was delivered into the cells using Oligofectamine (Invitrogen) (17).

**Western Blotting, Co-precipitation, Immunoprecipitation, and GST Pulldown Assay**—Cells were lysed in cell lysis buffer (Cell Signaling Technology) or SDS loading buffer. The lysates were resolved by SDS-PAGE and immunoblotted with appropriate antibodies. For co-precipitation, after transfection, whole cell lysates were extracted and the His-tagged proteins were precipitated with nickel-nitriotriacetic 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. For GST pulldown assay, pFLAG-CaMKII was transfected into LoVo cells, and the whole cell lysates were extracted. About 100 \(\mu\)g of cellular proteins was incubated with 100 \(\mu\)g of purified GST fusion proteins and glutathione-Sepharose 4B (Pierce) with or without 3 \(\text{mM}\) CaCl\(_2\). The precipitates were subjected to Western blot analysis.

**Cell Growth Assay**—The in vitro growth of transfected cells was measured by MTT dye reduction (18) and bromodeoxyuridine incorporation. For the in vivo evaluation of tumor growth, 2 \(\times\) 10\(^6\) LoVo or HT-29 cells were inoculated into BALB/c nu/nu athymic mice (4–5 weeks of age, BK Experimental Animal Co.) subcutaneously. When tumor grew to 2.5–3 mm in diameter, mice were randomly divided into three groups of six mice. Naked DNA was delivered into pre-established tumors by in vivo electroporation using an ECM 830 Square Wave Electroporation System (BTX) (18). Tumor growth was measured every 5 days. The tumor volume was calculated as follows: \(V = \frac{1}{2}(a \times b^2)\) (a, maximum tumor diameter; b, diameter at 90° to a) (19). Data were expressed as mean ± S.E., and analysis of variance was used for statistical analysis.

**Cell Cycle Analysis**—Cells were fixed with 70% ice ethanol, treated with 20 \(\mu\)g/ml RNase A (Sigma), stained with 20 \(\mu\)g/ml propidium iodide (Sigma), and analyzed by flow cytometry (FACSCalibur, BD Biosciences) for cell cycle distribution.

**Kinase Assay**—CaMKII kinase activity was assayed by CaMKII assay kit (New England Biolabs). For cdk2 kinase assay, 300 \(\mu\)g of cellular proteins was incubated with anti-cyclin A or cyclin E antibodies (Santa Cruz Biotechnology) pre-linked to protein G-Sepharose beads (Santa Cruz Biotechnology). The immunoprecipitates were subjected to kinase assay containing 5 \(\mu\)g of H1 histone (Calbiochem) and 10 \(\mu\)Ci of \([\gamma-32P]\)ATP (10 \(\mu\)Ci, Amersham Biosciences) for 15 min at 30 °C, and then SDS-PAGE. \([\gamma-32P]\)H1 histone was then visualized by autoradiography. Raf-1 and MEK1 activity was evaluated by Raf-1 kinase cascade assay kit and MEK1 assay kit (Upstate), respectively.

**Luciferase Assay**—Cells were transfected with p27PF or pGVB2 control vector, and 48 h post-transfection luciferase activity was determined using a Luciferase Reporter Assay kit (Clontech) according to the manufacturer’s instructions.
RESULTS

hCaMKIINα Is a Novel Human Endogenous CaMKII Inhibitory Protein—hCaMKIINα cDNA was identified by homology search and in silico assembly using human CaMKIINβ (hCaMKIINβ) cDNA sequence (16) and obtained by PCR amplification from bone marrow stromal cells. The hCaMKIINα cDNA encodes a 78-residue protein with a molecular mass of 8556.62 Da and an isoelectric point of 5.22. The novel protein shares high homology with the known CaMKII inhibitory proteins (CaMKIINs), including rat CaMKIINα (rCaMKIINα, 97% identity) (15), rCaMKIINβ (79% identity) (14), and hCaMKIINβ (65% identity) (16) (Fig. 1A). Based on its high homology with rat CaMKIINα and its inhibitory effect on CaMKII activity (see below), this novel protein was named as human CaMKII inhibitory protein alpha (hCaMKIINα). The full-length sequence was submitted into the NCBI GenBank database under accession number AY204901. Sequence analysis revealed a highly conserved 27-residue region in the C-terminal of CaMKIINs (Fig. 1A, represented by the solid line), which was referred to as 27-residue conserved inhibitory region (27CIR) throughout this report.

To characterize hCaMKIINα as a CaMKII inhibitor, we examined the interaction between both molecules. First, immunoprecipitation assay demonstrated the interaction between endogenously expressed hCaMKIINα and CaMKII (Fig. 1B). Second, by using a co-immunoprecipitation assay, we found that exogenously expressed hCaMKIINα and its 27CIR domain-truncated mutants (illustrated in Fig. 1C) all co-precipitated at different levels with CaMKII after ionomycin treatment (to elevate the calcium concentration and activate CaMKII), but not CaMKII without ionomycin stimulation (Fig. 1D). Finally, we performed a GST pulldown assay to further examine in vitro protein interaction. Purified recombinant GST-KIINα, GST-KIINα1–68, GST-KIINα1–53, GST-KIINα1–41, and GST-27tide, but not GST control, were bound to CaMKII in the presence of calcium (Fig. 1D) but not in the absence of calcium. These results suggest that the interaction of hCaMKIINα with CaMKII requires CaMKII activation by calcium, and that the 27CIR in hCaMKIINα is not essential but sufficient for this interaction.

To determine the effect of hCaMKIINα on CaMKII kinase activity, we used an in vitro kinase assay based on substrate phosphorylation by CaMKII. Phosphorylation of substrate by CaMKII was significantly suppressed by GST-KIINα, GST-KIINα1–68, GST-KIINα1–53, and GST-27tide proteins in a dose-dependent manner (Fig. 1E). The deletion of the last 10 residues (GST-KIINα1–68) did not obviously affect the inhibitory effect, and further truncation at residue 53 reduced its inhibitory capacity by 50–60%. However, GST-KIINα1–41 without the 27CIR did not exhibit any inhibitory effect. It’s of note that 27CIR alone effectively inhibited CaMKII activity. Autonomous CaMKII activity in cells was inhibited by overexpression of hCaMKIINα but increased by down-regulation of hCaMKIINα expression (p < 0.01, Fig. 1E). Similarly, pKIIα1–41 did not inhibit CaMKII activity while other mutants effectively inhibited CaMKII activity. Taken together, these results demonstrate that hCaMKIINα inhibits CaMKII activity in a dose-dependent manner and that the 27CIR plays a key role in its inhibitory capacity.

hCaMKIINα Expression Is Negatively Correlated with the Severity of Human Colon Adenocarcinoma—By determining the tissue and cellular expression of hCaMKIINα, we found hCaMKIINα in all the normal human tissues except skeletal muscle, with the highest expression in colon, small intestine, and ovary (Fig. 2A). hCaMKIINα is also expressed in several human solid tumor cells such as HT-29 (grade I adenocarcinoma) and LS-174T (grade II), but not in human colon adenocarcinoma cells LoVo (grade III), SW480, and SW620 (grade III) (Fig. 2A and B). This expression pattern is different from that of hCaMKIINβ, because hCaMKIINβ is not expressed in colon tissues and SW480 cells (16).

The expression pattern of hCaMKIINα also promoted us to further examine its expression in normal and neoplastic tissue specimens of human colon using anti-hCaMKIINα polyclonal antibody. To our interest, the results of tissue microarray showed that hCaMKIINα expression was inversely correlated with the severity (grade) of human colon adenocarcinoma (Fig. 2C). Generally, 66.2% (49/74) of colon adenocarcinoma samples were positive for hCaMKIINα expression, compared with 88.2% of margin tissue and 91.3% of normal colon samples (p < 0.0001 versus normal and margin group, Fig. 2D). In human colon adenocarcinomas, 86.7% (13 of 15) of grade I tumors were positive for hCaMKIINα expression, whereas 75.6% (31 of 41) of grade II tumors were positive. Strikingly, only 27.8% (5 of 18) of grade III tumors were positive for hCaMKIINα expression (p < 0.0001 versus normal, margin, grade I and grade II group), and the expression level was also relatively low. These results revealed a negative correlation of hCaMKIINα expression with the provenance and progression of human colon adenocarcinoma, suggesting that hCaMKIINα may be involved in the development of colon adenocarcinoma.

hCaMKIINα Suppresses Human Colon Adenocarcinoma Cell Growth Depending on Its Inhibition of CaMKII Activity—The negative correlation of hCaMKIINα expression with the severity of human colon adenocarcinoma led us to evaluate whether overexpression of hCaMKIINα affects tumor cell growth in vitro. Several human colon adenocarcinoma cells including LoVo, SW480, and SW620 cells (negative for endogenous hCaMKIINα expression), HT-29 and LS-174T cells (positive for hCaMKIINα expression) were transfected with hCaMKIINα expression plasmid, pKIIα, and then cell growth was analyzed by MTT assay. Western blot using anti-hCaMKIINα antibody confirmed the hCaMKIINα protein expression in the cell lines (supplemental Fig. S1A). The growth of LoVo (Fig. 3A), SW480 and SW620 cells (Fig. 3B) was markedly inhibited by hCaMKIINα overexpression (p < 0.05), whereas that of HT-29 and LS-174T cells was not significantly affected (Fig. 3B). The evaluation of cell proliferation by bromodeoxyuridine incorporation assay also confirmed the inhibitory effect of hCaMKIINα on the growth of tumor cells, which were negative for endogenous hCaMKIINα expression (Fig. 3C). We next assessed the effects of deleting endogenous
hCaMKIINα by using hCaMKIINα-specific siRNA (si-KIINα) in HT-29 and A549 cells (supplemental Fig. S1A). Down-regulation of hCaMKIINα expression markedly accelerated growth of HT-29 and A549 cells (Figs. 3A, 3C, and supplemental S2A, p < 0.05), suggesting that signaling through endogenous hCaMKIINα, not just exogenous hCaMKIINα, induced tumor growth suppression.

The role of the 27CIR in the growth inhibitory effect of hCaMKIINα was determined by domain truncation analysis. As demonstrated in Fig. 1E, the 27CIR was essential for the...
pKIINα was delivered into the established LoVo tumor in nude mice by *in vivo* electroporation. Immunohistochemistry analysis using anti-hCaMKIIα antibody confirmed hCaMKIIα protein expression in tumor tissues (supplementary Fig. S1B), indicating the effective *in vivo* delivery of pKIINα plasmid. In *in situ* expression of hCaMKIIα significantly inhibited the growth of established tumors *in vivo* (Fig. 3D, *p* < 0.05). On the other hand, purified hCaMKIIα siRNA-generating plasmid, pl-KIINα, was delivered into the established HT-29 tumor, and the accelerated tumor growth was observed (Fig. 3D, *p* < 0.05). Taken together, these findings indicate that hCaMKIIα inhibits tumor growth both *in vitro* and *in vivo*.

**hCaMKIIα-mediated CaMKII Inhibition Blocks Cell Cycle Progression by Up-regulating p27 Expression**—To cast light on the molecular mechanisms underlying the growth inhibition of hCaMKIIα in colon adenocarcinoma cells, we evaluated the effect of hCaMKIIα on cellular apoptosis and cell cycle progression. Overexpression of hCaMKIIα did not induce cellular apoptosis as assayed by Annexin V/PI staining followed by FACS analysis (data not shown). However, FACS analysis of cell cycle distribution showed an accumulation of cells in the S phase after transfection with pKIINα, pKIINα/\(1-68\), or pKIINα/\(1-53\) in LoVo cells, with a concomitant reduction in the number of cells in G1 phase (Fig. 4A), suggesting that the cells are arrested in the S phase and that the G1/S transition is not inhibited. Further confirming the regulation of cell cycle by hCaMKIIα, cell cycle analysis of HT-29 and A549 cells transfected with si-KIINα revealed decreased cell number in S phase (Fig. 4A and supplemental Fig. S2B). The result indicates that a cell-cycle checkpoint might be activated to block the completion of DNA replication in S phase.

To confirm the effect of hCaMKIIα on cell cycle progression, siRNA rescue assay was performed. si-KIINα-transfected HT-29 cells were transfected with a domain-truncated mutant of hCaMKIIα, pKIINα/\(1-53\), which would not be targeted by the synthetic siRNA. Cell cycle analysis revealed that overexpression of hCaMKIIα/\(1-53\) in hCaMKIIα-knockdown HT-29 cells could rescue the decrease in cell number of S phase (Fig. 4B), which further indicated the vital role of hCaMKIIα in S-phase progression. Therefore, it is the interference of the completion of DNA replication and then the S-phase progres-
Novel CaMKII Inhibitor Suppresses Tumor Growth

**FIGURE 3.** hCaMKINα inhibits tumor cell growth *in vitro* and *in vivo*. A, LoVo cells were transfected with hCaMKINα expression vectors or mock vectors, and HT-29 cells were transfected with si-KIINα or scrambled control (si-Non). In *in vitro* cell growth was examined by MTT assay as indicated. B, SW480, SW620, HT-29, and LS-174T cells were transfected with hCaMKINα expression vectors, and *in vitro* cell growth was examined by MTT assay 48 h after transfection. C, cell proliferation of LoVo cells transfected with hCaMKINα expression vectors and HT-29 cells transfected with si-KIINα were detected by FACS analysis of bromodeoxyuridine incorporation 48 h after transfection. D, the *in vivo* growth of LoVo and HT-29 cells after intratumoral expression or knockdown of hCaMKINα, respectively. Naked DNA of hCaMKINα expression vector pKIINα or siRNA-generating vector pKIINα was in *vivo* electroporated into established LoVo or HT-29 tumors, respectively, with empty vector as mock and PBS as control. *, p < 0.05 versus mock or control.

hCaMKINα expression and further confirms the blocking of endogenous hCaMKINα on S-phase progression.

We next explored the mechanism by which signaling through hCaMKINα could interfere the cell cycle progression. Cyclin/cyclin-dependent kinase complex plays an important role in regulating cell cycle progression. Cyclin E-cdk2 activity is required for progression from G1 to S phases; cyclin A-cdk2 activity is required for S progression (20). We then examined by kinase assay whether cyclin A- and/or cyclin E-associated cdk2 activity was affected by hCaMKINα. As shown in Fig. 4C, cyclin A-cdk2 activity was dramatically decreased in LoVo cells after transfection with pKIINα, pKIINα1–41, or pKIINα1–68, but not with pKIINα1–41 or mock plasmid, but cyclin E-cdk2 activity was not affected. The down-regulation of cyclin A-cdk2 activity prompted us to evaluate the levels of several cyclin kinase inhibitors in LoVo cells after overexpression of hCaMKINα. We observed that hCaMKINα expression led to significant up-regulation of p27, but not p21 (Fig. 4C). No change was found in pKIINα1–41-transfected cells, further confirming the key roles of 27CIR in hCaMKINα functions. On the other hand, down-regulation of hCaMKINα expression by siRNA in HT-29 cells decreased p27 expression (Fig. 4D). Moreover, we observed that cell growth was hardly affected by hCaMKINα overexpression in p27 siRNA (si-p27)-transfected LoVo cells (Fig. 4E), further confirming the key role of p27 in hCaMKINα-mediated inhibition of cell growth. Taken together, our findings suggest that hCaMKINα-mediated CaMKII inhibition, through its 27CIR, causes the up-regulation of p27 expression, resulting in the arrest of S phase progression.

hCaMKINα-induced p27 Accumulation Is Attributed to the Repressed Proteasomal Degradation, but Not Transcriptional Enhancement, of p27—We then investigated the possible mechanisms that contributed to hCaMKINα-induced p27 up-regulation: transcription or degradation regulation. Both the p27 promoter activity and the basal p27 transcript were barely changed in hCaMKINα-transfected LoVo cells (Fig. 5A). However, treatment of MG132, a specific proteasome inhibitor, fully reversed the down-regulation of endogenous p27 (Fig. 5B) and...
exogenously expressed FLAG-tagged p27 protein (Fig. 5C) induced by si-KIINα in HT-29 cells. Besides, in LoVo cells treated with cycloheximide (a ribosomal complex inhibitor, to inhibit protein synthesis), p27 level decreased gradually as time passed; whereas hCaMKIINα overexpression inhibited the reduction of p27 protein (Fig. 5D). In the constitutively active form of CaMKII (H282R)-transfected LoVo cells, decreased p27 expression was also observed (data not shown). Together, the data show that hCaMKIINα-mediated CaMKII inhibition suppresses the proteasome-mediated degradation, resulting in the stabilization and therefore the accumulation of p27.

MEK/ERK De-activation Promotes p27 Stabilization in hCaMKIINα/CaMKII Signaling—Because only phosphorylated p27 on Thr-187 can be recognized and bound by ubiquitin protein ligase for consequent ubiquitination and degradation (21), we therefore tested whether p27 phosphorylation was affected by hCaMKIINα. The results showed that phosphorylation on p27(T187) was decreased in LoVo cells transfected with pKIINα, pKIINα1–41, and pKIINα1–68, but not pKIINα1–53 or mock control (Fig. 6A), and the expression of a Thr-187 mutant, T187A p27, was not affected by hCaMKIINα overexpression (data not shown), suggesting that p27(T187)
phosphorylation accounting for its degradation may be decreased by hCaMKIIα-mediated inhibition of CaMKII activity, and that CaMKII may promote p27 phosphorylation directly or indirectly. However, in vitro kinase assays showed that CaMKII could not directly phosphorylate p27 protein (data not shown). So, which of the one or more signal pathways are involved in the inhibition of p27 phosphorylation mediated by hCaMKIIα-induced CaMKII inhibition? We considered first the MAPK and phosphatidylinositol 3-kinase/Akt pathways (22, 23). We found that the phosphorylation of MEK1 and ERK1/2, but not that of p38, JNK, or Akt, was decreased in HT-29 cells co-transfected with p27PF and control pGVB2 plasmid together with pKIINα. 48 h post-transfection cells were harvested and lysed for in vitro reporter assay for luciferase activity. The means ± S.E. of three independent experiments are indicated (left panel). p27 mRNA expression was analyzed by quantitative real-time PCR using specific primer sets for p27 and β-actin in pKIINα-transfected LoVo cells 48 h post-transfection (right panel). The relative expression of p27 to β-actin and S.E. are indicated. B, si-KIINα-transfected HT-29 cells were treated with MG132 (10 μM) or DMSO 2 h before harvest. 48 h post-transfection the cell lysates were subjected to Western blot with the indicated antibodies. C, HT-29 cells were co-transfected with FLAG-p27 and si-KIINα, and treated with MG132 or DMSO 2 h before harvest. 48 h post-transfection cells were lysed and subjected to Western blot (WB) with the indicated antibodies. D, LoVo cells transfected with pKIINα or mock vector were incubated with cycloheximide (10 μM) for the indicated time before harvest. 48 h post-transfection cells were lysed and subjected to Western blot with the indicated antibodies.

If the hCaMKIIα and MAPK pathways converge upstream of MEK/ERK to regulate p27 phosphorylation, this leaves Raf-1 or MEK as possible targets for CaMKII and hCaMKIIα regulation. We then examined whether Raf-1 and MEK1 activities were affected by hCaMKIIα overexpression. The result showed that hCaMKIIα induced a significant decrease in the MEK1 activity, but not Raf-1 activity, as compared with mock-transfection (Fig. 6C), demonstrating that hCaMKIIα regulates MAPK signaling at the level of MEK1. Besides, U0126 (MEK1 inhibitor) treatment nearly completely abrogated the increased phosphorylation of endogenous ERK1/2 and p27(T187) in HT-29 cells transfected with si-KIINα (Fig. 6D). Collectively, the data indicate that the deactivation of MEK/ERK signaling by hCaMKIIα may be responsible for the inhibition of p27 phosphorylation and thereafter degradation, which primarily regulates the cell cycle progression of tumor cells.

**DISCUSSION**

Advanced colorectal adenocarcinoma is generally poorly responsive to chemotherapy and radiation. To improve treatment outcomes, a better understanding of the pathways that underlie the behavior of colorectal cancers and contribute to deregulated proliferation is necessary. CaMKII inhibitors are potential candidates for regulating these events. Exogenous synthetic CaMKII inhibitors such as KN-62, KN-93, and AIP have been found to induce cell cycle arrest or cellular apoptosis in a variety of malignant cells such as K562 and HeLa (5, 8, 12). Also, our previous study showed that endogenous human CaMKII inhibitor protein (hCaMKII), hCaMKIIα, significantly inhibits LoVo cell proliferation (16). By identifying another novel endogenous hCaMKII, hCaMKIIα, and using human colon adenocarcinoma cells as tumor model, we demonstrate that the effective suppression of LoVo cell growth by hCaMKIIα is due to a dramatic inhibition of cell-cycle progression with features of an S-phase arrest. In this case, hCaMKIIα caused deactivation of the kinase activity of MEK/ERK and the accumulation of p27 protein, which primarily regulates the cell cycle progression of LoVo cells. This is the first report on mechanistic details underlying how endogenous CaMKII inhibitor-mediated CaMKII inhibition arrests cell cycle in tumor cells.

We have demonstrated that the target of hCaMKIIα-mediated CaMKII inhibition is more like to be p27. As we all know, p27 can bind to cyclin A/Cdk2 and cyclin E/Cdk2 and inhibit its activity. Cyclin E-cdk2 activity is required for progression from G1 to S phases, and cyclin A-cdk2 activity is required for the S progression (20). In our study, the inhibition of cyclin A/Cdk2 activity but not cyclin E/Cdk2 activity was observed. We also observed the decrease of cyclin A protein level by hCaMKIIα overexpression, but the level of cyclin E remained unchanged. Although cyclin E levels decrease rapidly once S phase is initiated in normally growing cells (24), previous studies show that early inhibition of p27 phosphorylation, which occurred at 16 h and became more significant at 24 h (Fig. 6B and supplemental Fig. S5).

4. C. Wang, N. Li, X. Liu, Y. Zheng, and X. Cao, unpublished data.
inhibition of Cdk2 activity leads to cyclin E stabilization by inhibiting autophosphorylation and subsequent ubiquitin-dependent degradation of cyclin E (25). Thus, the unaltered expression of cyclin E in LoVo cells overexpressing hCaMKIINα might also reflect the inhibition of Cdk2 activity in late G1 phase.

Because CaMKII is a multifunctional serine/threonine protein kinase that can phosphorylate various substrate proteins ubiquitously, and p27 Thr-187 phosphorylation accounting for its degradation may be decreased by hCaMKIINα, we wonder whether p27 may act as physiological substrate of CaMKII. However, in vitro kinase assay showed that CaMKII could not directly phosphorylate p27 protein (data not shown). Sequence analysis also showed that CaMKII phosphorylation motifs were not present in the sequences of p27 protein. The results suggest that hCaMKIINα may affect p27 expression indirectly via other target signaling molecule(s). First we considered the signaling molecules that account for associated properties of p27 in negative regulation of cell-cycle progression, such as MAPK and phosphatidylinositol 3-kinase/Akt pathways (22, 23). We found that the hCaMKIINα-mediated deactivation of MEK1/2 and ERK1/2, but not that of p38, JNK or Akt, is prerequisite to the inhibition of phosphorylation and subsequent proteasomal degradation of p27 (Fig. 6). Although previous studies showed that CaMKII could activate Ras/Raf-1/MAPK pathway by binding to and activating Raf-1 (26, 27), the Raf-1 activity was not affected by hCaMKIINα (Fig. 6C), suggesting that Raf-1 might not be a direct target of hCaMKIINα effect. However, MEK kinases other than Raf-1 may be involved in the interaction with CaMKII (28). Nevertheless, we are the first to report the hCaMKIINα-induced deactivation of MEK/ERK activity leads to p27 modulation and cell cycle regulation. We are now working on the potential phosphorylation of p27 and the exact phosphorylation site(s) of p27 by MEK/ERK.

Most of the known genetic mechanisms correlated with the tumor progression involve the loss of growth inhibitory functions of wild-type suppressor genes, leading to unregulated or malignant growth. A number of biological consequences with clinical relevance arise from the MAPK mis-regulation. Of particular interest here are the absence of hCaMKIINα in poorly differentiated tumors and its inhibition of human colon carcinoma growth as a candidate tumor suppressor. Besides, p27 has long been evaluated as negatively correlated with advanced tumor stage and short survival of patients with colon cancers.
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(29, 30). In addition, constitutive activation of MEK1/2 has been found in tumorigenesis. We observed a negative correlation of hCaMKIIα expression with p27 in several colon cancer cell lines (supplemental Fig. S6), and our preliminary results showed that, in some of the matched normal and tumor samples, p27 expression is positively correlated with hCaMKIIα expression. Moreover, the low expression of hCaMKIIα in colon adenocarcinoma was found to be negatively correlated with MEK/ERK activation status (p < 0.05). Thus, the likelihood of a contributory role for hCaMKIIα in the tumor development is further strengthened by its involvement in the induction of p27 accumulation, and subsequent cell cycle interference. These data led us to hypothesis that the deregulation of hCaMKIIα in colon adenocarcinoma tissues could be impairing a potential critical step to cell cycle progression, thereby favoring tumor cell development and growth. So, we speculate that amplifying hCaMKIIα-mediated CaMKII inhibition may provide a promising approach for the drug design of cancer therapeutics.

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