Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II Promotes Cell Cycle Progression by Directly Activating MEK1 and Subsequently Modulating p27 Phosphorylation*5

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Cell proliferation is regulated by integration of multiple pathways, such as MAPK, phosphatidylinositol 3'-kinase, protein kinase C, and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) signaling, determining whether the cell proceeds into cell cycle progression. Recently, we have demonstrated that a novel endogenous CaMKII-inhibitory protein, hCaMKII\(\text{I}\)\(\alpha\), suppresses tumor growth by inducing cell cycle arrest via p27 stabilization, accompanied by MEK/ERK deactivation. The data indicate a potential link between Ca\(^{2+}\)/CaMKII and other signaling pathways, such as MAPK signaling. However, the detailed mechanisms of cross-talks between these important pathways on cell cycle regulation have not been specified. Here we report that CaMKII, in colon adenocarcinoma cells, activates MEK/ERK, which is responsible for the phosphorylation and subsequent proteasomal degradation of p27, thus causing the promotion of the S-G\(_{2}/M\) transition of cell cycle progression. Importantly, we found that CaMKII can bind to MEK1 and that active CaMKII directly phosphorylates MEK1 in vitro, which could be abrogated by CaMKII inhibitor. Besides, ERK2 can directly interact with and phosphorylate p27. This is the first demonstration that CaMKII interplays with MEK1 and regulates p27 phosphorylation in the cell cycle progression. These findings provide mechanistic evidence for the cross-talk between CaMKII and MAPK signaling, which converges in MEK/ERK activation in the regulation of cell cycle progression.

Coordinate regulation of intracellular signaling pathways is essential for mitogens and oncogenes to promote cell cycle progression (1). Several pathways are thought to play important roles in committing cells into cell cycle, including the mitogen-activated protein kinase (MAPK),4 phosphatidylinositol 3’-kinase/Akt, protein kinase C, and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) pathways (2, 3), etc. These pathways are reported to influence the expression, activity, or subcellular localization of key components of the cell cycle machinery, such as cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors, leading to the appropriate activation of E2F transcription factors (4). Evidence for signal pathway cooperation to attain the appropriate extent and timing of response has been described in a number of different situations. For example, in colon cancer cells, Wnt pathway cooperates with activated RAS to regulate the expression of cyclin D1 to promote S-phase progression (5). In model systems, RAF and phosphatidylinositol 3’-kinase pathways can cooperate in oncogenic transformation and in promotion of the reentry into the cell cycle (6, 7).

Members of the Ca\(^{2+}\)/calmodulin -dependent protein kinase family are major biochemical decoders of intracellular Ca\(^{2+}\) oscillations, among which CaMKII is critical for many physiological and pathological functions of cells (8, 9). Recent observations clearly suggest a potential link between CaMKII and cell cycle regulation. A synthesized chemical CaMKII inhibitor, KN-93, could decrease cyclin-dependent kinase 4 activity by reducing cyclin D1 levels and cyclin-dependent kinase 2 activity by enhancing p27\(^{\text{Kip1}}\) (p27) expression, causing cell cycle arrest at the G\(_{1}\) phase (10). Our recent results showed that a novel endogenous CaMKII-inhibitory protein, hCaMKII\(\text{I}\)\(\alpha\), suppresses tumor growth by inducing cell cycle arrest via p27 stabilization (11). However, components of the signaling pathway that lie between the Ca\(^{2+}\)/CaMKII signaling and the cell cycle machinery have been undefined. This is particularly relevant, since potential connections between Ca\(^{2+}\)/CaMKII signaling and multiple signaling pathways have been reported in many cell types; elevated extracellular Ca\(^{2+}\) can activate the MAPK pathways (12), the CaMKII-MAPK pathways regulate the neuronal cell fate determination (13), and the Wnt-5a-mediated Ca\(^{2+}\) signaling activates the TAK1-NLK pathway via CaMKII (14). Our results also show that hCaMKII\(\text{I}\)\(\alpha\) deactivates MEK/ERK and causes the inhibition of S-phase progression of the cell cycle (11). This raises the possibility that CaMKII acts upstream

4 The abbreviations used are: MAPK, mitogen-activated protein kinase; CaMKII, calcium/calmodulin-dependent protein kinase II; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; GST, glutathione S-transferase.
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to activate the MAPK pathway in the regulation of cell cycle progression. However, the detailed mechanisms of the cross-talk between CaMKII and MAPK pathways for the regulation of cell cycle have not been specified to date.

Thus, the primary aim of this study is to investigate the potential signaling interaction between CaMKII and MAPK pathways in the regulation of cell growth and cell cycle progression. We showed that CaMKII activated MEK/ERK to enhance p27 (Thr187) phosphorylation and subsequent degradation, resulting in rapid entry of tumor cells into G2/M phase. Importantly, we found that CaMKII can bind to MEK1 and that active CaMKII can directly phosphorylate MEK1 in vitro, which could be abrogated by CaMKII inhibitor. Besides, ERK2 can directly interact with p27 in cells and phosphorylate p27 (Thr187) in vitro. Our findings provide a new mechanistic model for the cross-talk between CaMKII and MAPK signaling, which converges in MEK/ERK activation to regulate p27 stabilization and thus promote cell cycle progression.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Antibodies—LoVo and SW620, human colon adenocarcinoma cells, obtained from ATCC, were maintained in F-12 or RPMI 1640 medium (Invitrogen), respectively, containing 10% fetal bovine serum. MG-132 and U0126 were from Calbiochem. Anti-p27, phosphorylated p27 (Thr187), and MEK1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-ERK1/2, phosphorylated MEK1, and phosphorylated ERK1/2 antibodies were from Cell Signaling Technology; and anti-FLAG, CaMKIIα, and actin antibodies were from Sigma.

Plasmid Construction and Cell Transfection—FLAG-tagged expression vectors of CaMKIIα with His328 mutated to Arg (H282R) were constructed by PCR cloning and PCR mutation (11, 15). The sequences encoding amino acids residues 1–154 (designated YN154) or 155–238 (designated YC155) of enhanced yellow fluorescent protein were fused to the 5′-ends of the coding regions for ERK2 or p27 by using AAANSSDILS-VPVDSR linkers (16). The chimeric coding regions were cloned into pcDNA3.1/myc-His(−)B to produce plasmids encoding ERK-YN and p27-YC. Vectors were transfected into cells by Lipofectamine2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cells were subjected to analysis 48 h post-transfection except as specified.

Cell Growth Assay—The in vitro growth of transfected cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay and bromodeoxyuridine incorporation (11).

Cell Cycle Analysis—Transfected LoVo cells were fixed with 70% ice ethanol, treated with 20 μg/ml RNase A (Sigma), stained with 20 μg/ml propidium iodide, and analyzed by flow cytometry (FACSCalibur; BD Biosciences) for cell cycle distribution.

Kinase Assay—CaMKII kinase activity was assayed by a CaMKII assay kit (New England Biolabs), and [γ-32P]ATP was from Amersham Biosciences. For CaMKII-mediated phosphorylation of MEK1, recombinant inactive MEK1 (Upstate) or immunoprecipitated MEK1 from cell lysates was used as CaMKII substrate in the above CaMKII kinase assay system.

For the phosphorylation of p27 by ERK, immunoprecipitated ERK1/2 or recombinant active ERK2 (Calbiochem) was incubated with recombinant p27 (Santa Cruz Biotechnology) in a kinase reaction. The products were subjected to scintillation counting or, alternatively, subjected to Western blot using anti-phosphorylated p27 (Thr187) antibody.

Western Blotting Co-precipitation, and Immunoprecipitation—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-nitrilotriacetic 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.

Bimolecular Fluorescence Complementation—Cells grown on glass coverslips were transfected with plasmids encoding ERK-YN or p27-YC fusion proteins. 36 h post-transfection, cells were stained with Hoechst 33258 (5 μg/ml; Sigma). Fluorescence images were then collected by fluorescence confocal microscopy (LSM confocal microscope; Carl Zeiss) (16).

Statistical Analysis—All data were presented as means ± S.E. Statistical analysis (Student’s t test) was performed using the computer program SPSS version 6.1.

RESULTS

CaMKII Promotes the Growth and S-G2/M Transition and Enhances the Phosphorylation and Degradation of p27 in Human Colon Adenocarcinoma Cells—Our previous study showed that hCaMKINα, an endogenous CaMKII inhibitory protein, as well as KN-62, a chemical inhibitor of CaMKII, inhibited the growth of colon cancer cells (11). Here we constructed a vector expressing a constitutively active form of CaMKII, H282R (15), and observed whether the overexpression of active CaMKII affected colon tumor cell growth in vitro. Western blot using anti-FLAG antibody confirmed the H282R protein expression in the transfected cells (Fig. S1A). As shown in Fig. 1A, the growth of LoVo and SW620 cells transfected with H282R were accelerated compared with mock or parental cells (p < 0.05) as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, which was also confirmed by evaluation of cell proliferation by a bromodeoxyuridine incorporation assay (Fig. 1B). To begin to explore the mechanisms by which CaMKII promotes cell growth, we evaluated the effect of CaMKII on cellular apoptosis and cell cycle progression. Over-expression of H282R did not induce cell apoptosis, as assayed by annexin V/PI staining, followed by FACS analysis (data not shown). However, FACS analysis of cell cycle distribution revealed a reduction of cells in the S phase, with a concomitant accumulation in the number of cells in G2/M phase compared with mock or parental cells (Fig. 1C and Fig. S1B). The percentages of the cells in the G2/M phase were increased by 13.39% (p < 0.01 compared with mock), suggesting that CaMKII promotes S-G2/M transition of cell cycle progression, which is responsible for the growth promotion of colon adenocarcinoma cells by CaMKII.
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p27, a key regulator of progression from G₁ to S phase, is often functionally inactivated in a majority of human cancers. A synthesized chemical CaMKII inhibitor, KN-93, could enhance p27 expression, causing cell cycle arrest at the G₁ phase in HeLa cells (10). The endogenous CaMKII inhibitory protein, p27, expression, causing cell cycle arrest at the G₁ phase in HeLa cells, p27 expression was markedly decreased compared with mock or parental cells (Fig. 2A and Fig. S2A). These data revealed that p27 (Thr<sup>187</sup>) phosphorylation accounting for its degradation may be increased by CaMKII activation. Although our previous data showed that CaMKII could not directly phosphorylate p27 protein (11), the phosphorylation on Thr<sup>187</sup> of p27 derived from immunoprecipitated complex by anti-p27 immunoprecipitation could be dramatically increased by CaMKII, as determined by an in vitro kinase assay (Fig. 2C and Fig. S2C). These results suggested that CaMKII could indirectly phosphorylate p27 (Thr<sup>187</sup>), resulting in p27 degradation.

MEK/ERK Activation Is Required for CaMKII-induced p27 (Thr<sup>187</sup>) Phosphorylation—It is well accepted that multiple pathways, such as MAPK and phosphatidylinositol 3′-kinase/Akt, are involved in p27 phosphorylation (18–21). Since CaMKII could not directly phosphorylate p27, we needed to determine which signal pathway was involved in the regulation of p27 phosphorylation mediated by CaMKII. We first evaluated the activation of Akt and MAPK after H282R overexpression. We found that the phosphorylation of MEK1/2 and ERK1/2 were increased in H282R-transfected cells compared with mock or parental cells (Fig. 3A and Fig. S3); however, the levels of p38, c-Jun N-terminal kinase, and Akt phosphorylation were barely affected (data not shown). Besides, CaMKII could increase the phosphorylation of exogenously expressed MEK1 (Fig. 3B). To confirm that the MEK/ERK cascade is involved in CaMKII activation-mediated p27 (Thr<sup>187</sup>) phosphorylation, we treated cells with the MEK1 inhibitor, U0126. U0126 treatment resulted in a dramatic elimination of the Thr<sup>187</sup> phosphorylation of both endogenously and exogenously expressed p27 (Fig. 3A), similar to that observed upon CaMKII inhibition by hCaMKII5 (11). When cells were transfected with H282R, active CaMKII-induced p27 (Thr<sup>187</sup>) phosphorylation was nearly completely abrogated by U0126 (Fig. 3A and Fig. S3), suggesting that MEK/ERK activation is a prerequisite for the phosphorylation of p27 (Thr<sup>187</sup>) induced by CaMKII activation. Collectively, the data indicate that activation of MEK/ERK cascade by CaMKII may be responsible for p27 phosphorylation and thereafter degradation, which primarily...
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FIGURE 3. CaMKII modulates p27 phosphorylation through MEK/ERK signaling. A, LoVo cells were transfected with constitutively active CaMKII (H282R) or empty vector. 24 h post-transfection, the cells were treated with U0126 (20 µM) for 24 h, lysed, and subjected to Western blot with the indicated antibodies (left). Alternatively, LoVo cells were transfected with FLAG-tagged p27 expression vector (FLAG-p27) or empty vector. 24 h post-transfection, the cells were treated with U0126 (20 µM) for 24 h, lysed, and subjected to Western blot with the indicated antibodies (right). B, the effect of CaMKII on the phosphorylation of exogenously expressed MEK1. LoVo cells were co-transfected with H282R and His-tagged MEK1 vector (His-MEK1). 48 h post-transfection, the exogenously expressed His-tagged MEK1 was co-precipitated (CP) using Ni²⁺-nitrotriacetic acid (Ni-NTA) beads and Western blot (WB) was then performed with the indicated antibodies.

FIGURE 4. CaMKII associates with and directly phosphorylates MEK1. A, interaction between endogenous CaMKII and MEK1. Lysates of LoVo cells were immunoprecipitated (IP) with anti-MEK1 (left) or anti-CaMKII (right) or control IgG. The immunoprecipitates were analyzed by Western blot with anti-MEKKI or anti-MEK1, respectively. B and C, CaMKII phosphorylated MEK1 in vitro. Recombinant unactive MEK1 and immunoprecipitated MEK1 were used as substrates in the presence or absence of GST-KIINα in an in vitro kinase reaction. The products were then subjected to Western blot analysis (B) or scintillation counting (C) for MEK1 phosphorylation. **, p < 0.01; ***, p < 0.005.

The Cross-talk between CaMKII and MAPK Signaling Converges at the MEK1 Level—We then investigated the interplay between CaMKII and MEK/ERK signaling. Recent study showed that CaMKII could phosphorylate MEK by binding to and activating Raf-1. However, our previous study showed that overexpression of hCaMKIINα induced a significant decrease in the MEK1 activity but not Raf-1 activity (11), suggesting that CaMKII might not regulate MAPK signaling at the Raf-1 level. To further determine the relationship between CaMKII and MEK/ERK signaling, we investigated whether CaMKII physically interacts with MEK1 in intact cells. As shown in Fig. 4A, CaMKII protein could be detected in anti-MEK1 immunoprecipitates (Fig. 4A, left). In a reciprocal experiment, MEK1 protein was also found in anti-CaMKII immunoprecipitates (Fig. 4A, right). Therefore, an association between endogenous CaMKII and MEK1 was detected, indicating that CaMKII and MEK1 interact with each other in vivo.

We next tested whether CaMKII phosphorylates MEK1. Extracts of LoVo cells were subjected to immunoprecipitation with anti-MEK1 antibody, followed by both in vitro kinase assay and immunoblotting. The results showed that, when activated CaMKII was present in the kinase reaction, MEK1 became phosphorylated (Fig. 4B), suggesting that activated CaMKII induces phosphorylation of MEK1. Furthermore, we also detected a direct phosphorylation of unactive recombinant MEK1 protein (but not purified ERK protein; data not shown) by CaMKII in vitro (Fig. 4B). Thus, the results indicate that MEK1 may be a direct substrate of CaMKII in cells.

We then determined the effect of CaMKII on MEK1 activity. To this end, endogenous MEK1 protein was immunoprecipitated from the cell lysates with anti-MEK1 antibody, and its kinase activity was measured with MBP as a substrate. MEK1 activity was enhanced when activated CaMKII was present in the kinase reaction (Fig. 4C), which was abrogated by purified hCaMKIINα protein, GST-KIINα (Fig. 4C), but not GST control protein (data not shown). Moreover, we found that activated CaMKII protein could also phosphorylate inactive recombinant MEK1 protein (but not recombinant ERK protein) in vitro, which was also abrogated by GST-KIINα protein (Fig. 4C). These results support the hypothesis that CaMKII leads to MEK1 activation and indicate that the cross-talk between CaMKII and MAPK signaling converges at the level of MEK1.

Activation of MEK/ERK Cascade Directly Phosphorylates p27—To investigate the downstream signaling of MEK/ERK in CaMKII-induced p27 degradation, we tested the effect of MEK1 on the p27 (Thr^{187}) phosphorylation. We found that exogenous expression of constitutively active MEK1 (MEK-A) regulates the cell cycle progression of human colon adenocarcinoma cells.
resulted in the activation of ERK1/2 as well as the Thr\(^{187}\) phosphorylation of both endogenous and exogenous p27 (Fig. 5A and Fig. 5A, A and B). These results suggest that activation of the MEK/ERK cascade induces p27 (Thr\(^{187}\)) phosphorylation.

It was reported that FAR1, a yeast cyclin-dependent kinase inhibitor, is phosphorylated by FUS3, a member of the MAPK family (22), so we then examined the p27 phosphorylation by MAPK in vitro. A purified recombinant p27 was used as substrate in kinase reactions. Anti-ERK1/2 immunoprecipitates were able to phosphorylate recombinant p27 protein, but normal IgG immunoprecipitates or no anti-ERK control could not (Fig. 5, B and C). To eliminate the possibility that some contaminating kinase(s) in the immunoprecipitates phosphorylated p27, we conducted an experiment with a purified recombinant ERK2 protein and found that the recombinant ERK2 protein could also phosphorylate p27, at least at Thr\(^{187}\) (Fig. 5, B and C), thus convincingly demonstrating that p27 is directly phosphorylated by ERK in vitro.

Finally, we analyzed the interaction between ERK1/2 and p27. As shown in Fig. 5D, a substantial amount of ERK1/2 was detected in an anti-p27 immunoprecipitates (Fig. 5D, left). Reciprocally, we found a significant portion of p27 protein in an anti-ERK1/2 immunoprecipitates (Fig. 5D, left). Therefore, the association between ERK1/2 and p27 was detected. To further visualize the physical interaction between ERK and p27 in living cells, we used the bimolecular fluorescence complementation assay, which is based on complementation between nonfluorescent fragments of the enhanced yellow fluorescent protein when brought together by an interaction between proteins fused to each fragment (16). The N-terminal fragment (residues 1–154) of yellow fluorescent protein was fused to ERK2 (ERK-YN), and the C-terminal fragment (residues 155–238) to p27 (p27-YC). We transfected ERK-YN and p27-YC expression vectors alone or together into SW620 cells and then examined the cells by confocal fluorescence microscopy. When expressed alone, neither
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ERK-YN nor p27-YC produced detectable fluorescence (Fig. 5E). The expression of the fusion proteins in cells that did not display fluorescence signals was confirmed by Western blot using anti-yellow fluorescent protein (N-terminal or C-terminal) antibodies (data not shown). However, once co-transfected into cells, a strong yellow fluorescence signal was visualized and associated preferentially with the nucleus 36 h post-transfection (Fig. 5E), indicating that ERK-YN-p27-YC heterodimer was formed to generate visualized yellow fluorescence and that the heterodimer localized in nucleus. The results suggest that ERK and p27 physically interact with each other in cells.

DISCUSSION

Although the cross-talk between Ca²⁺/CaMKII and other signaling pathways has been implied by several lines of evidence, the signaling mediators that play important cooperative roles in mitogenic Ca²⁺/CaMKII signaling have not been specified. Our studies add a new layer of complexity to the functions of CaMKII in diverse signaling networks that contribute to the tumor progression. We have demonstrated here that CaMKII can directly bind to MEK1, activate the kinase activity of MEK/ERK, and enhance the phosphorylation of p27 protein, therefore promoting the S-G₂/M transition of the cell cycle progression and then the tumor cell growth. It seems likely that the integration of signals from multiple kinase cascades within a particular cell, such as described here for CaMKII and MAPK, will play an important role in mediating the specificity and biological outcome of signal transduction pathways.

We propose here a mechanistic model by which CaMKII promotes cell cycle progression in tumor cells (Fig. 6). In this model, upon mitogenic stimuli, such as treatment with sera and mitogens, CaMKII is activated by decoding the signals of increased intracellular Ca²⁺ and interacts with MEK1, leading to the activation of MEK1 and subsequent activation of ERK1/2. Activated ERK (directly or indirectly) phosphorylates p27 at Thr¹⁸⁷, allowing p27 for SCF⁶⁵² ubiquitin-protein ligase recognition (23) and subsequent ubiquitin-proteasome-dependent degradation in the S phase. As a result, the tumor cells are enhanced to go through S-G₂/M transitions of the cell cycle and therefore rapid cell proliferation. Thus, Ca²⁺/CaMKII signaling converges with the MAPK pathway, at least partially in the regulation of MEK/ERK activity, to control cell cycle progression. The model may provide a possible mechanistic explanation for the cell cycle arrest induced by CaMKII inhibitors (such as hCaMKIINα); i.e. hCaMKIINα-mediated CaMKII inhibition may disrupt this cross-talk, causing the deactivation of the kinase activity of MEK/ERK and the accumulation of p27 protein, which primarily regulates the cell cycle progression of tumor cells.

An important issue we addressed in this model is the cross-talk between CaMKII and MAPK signaling, which promotes p27 phosphorylation. It has been shown that activation of protein kinase C, ERK, p38, or c-Jun N-terminal kinase/stress-activated protein kinase plays vital roles in Ca²⁺/CaMKII-dependent cellular response to mitogenic signals and inflammatory stimuli in many cell types, including vascular smooth muscle cells (24), cultured rat cortical neurons (25), and macrophages (26, 27), and inhibition of CaMKII represses the activation of those signal pathways. Accordingly, we examined the activation of phosphatidylinositol 3'-kinase/Akt and MAPK in LoVo cells after CaMKII overexpression and found that activated CaMKII increased the phosphorylation of MEK1 and ERK1/2 but not those of Akt, p38, and c-Jun N-terminal kinase. Moreover, MEK inhibitor U0126, but not phosphatidylinositol 3'-kinase, p38 or protein kinase C inhibitors, prevented the up-regulated phosphorylation and therefore the degradation of p27 in LoVo cells with constitutively active CaMKII expression. These data demonstrate that p27 phosphorylation induced by CaMKII is dependent on the activation of MEK/ERK and that the MEK/ERK cascade is a key regulator that links the CaMKII signaling to cell cycle regulation. Although previous studies showed that CaMKII could activate Ras/Raf-1/MAPK pathway by binding to and activating Raf-1 (28, 29), the regulation of CaMKII-binding Raf-1 was barely detected in our studies (data not shown), and the Raf-1 activity was not affected by hCaMKIINα overexpression (11), suggesting that Raf-1 might not be a direct target of hCaMKIINα-mediated CaMKII inhibition. Instead, we found that CaMKII and MEK1 associated with each other in LoVo cells. Although a direct MEK1 phosphorylation by CaMKII has not been described before, the existence of seven canonical (R/K)XX(S/T) consensus sequences for CaMKII makes MEK1 phosphorylation by CaMKII a possibility worthy of consideration. Indeed, our in vitro experiments demonstrate that both recombinant and immunoprecipitated MEK1 (but not recombinant ERK protein) can be directly phosphorylated by CaMKII, and the phosphorylation could be abolished by the addition of hCaMKIINα protein (Fig. 4). Our results demonstrate for the first time that MEK1 is a target of CaMKII in the pathway that regulates p27 phosphorylation downstream in the cell cycle machinery.

**FIGURE 6. Models depicting the roles of MEK/ERK signaling in CaMKII-induced promotion of cell cycle progression.** Activation of CaMKII phosphorylates MEK1, leading to ERK activation. The phosphorylation of p27 (Thr¹⁸⁷) by ERK is enhanced. Hyperphosphorylation of p27 results in p27 degradation by the ubiquitin-proteasome pathway and therefore leads to down-regulation of p27, inducing rapid entry of tumor cells into G₂/M phase.
Although this does not demonstrate that MEK1 is a CaMKII substrate in vivo, it supports this possibility. Therefore, a direct link between CaMKII and the MEK/ERK cascade is established in our experimental system.

Next, another unexpected result gave a possible answer to how the MEK/ERK cascade affects p27 phosphorylation. The p27 protein contains four consensus sites (Ser10, Ser40, Ser178, and Thr187) that form the core of the phosphorylation site by MAPK (30, 31). We found that immunoprecipitated ERK1/2 protein contains four consensus sites (Ser10, Ser40, Ser178, and Thr187) that form the core of the phosphorylation site by MAPK (30, 31). We found that immunoprecipitated ERK1/2 and recombinant ERK2 could directly phosphorylate p27 protein in vitro (Fig. 5B). Similar to our findings, it has been previously reported that ERK1/2 phosphorylates p27 in vitro (32, 33). More directly, we detected association between ERK1/2 and p27 by immunoprecipitation as well as physical interaction between ERK and p27 in living cells by a bimolecular fluorescence complementation assay (Fig. 5D). Although we have not yet compared relative phosphorylation level and timing of p27 by ERK with other intracellular substrates of MAPK, it is likely that the sustained activation of ERK after the transient activation should be required to degrade p27. Provided that CaMKII-mediated MEK activation and therefore ERK activation phosphorylates p27 at Thr187 in vivo, it is tempting to assume that phosphorylation of this amino acid allows for SCFSkp2 ubiquitin-protein ligase recognition and subsequent ubiquitin-proteasome-dependent p27 degradation, resulting in the promotion of cell cycle progression and cell proliferation (Fig. 1). However, the in vitro phosphorylation of p27 by ERK might not be the case in vivo, and we cannot rule out the possibility that MAPK kinase kinases (MEKKs) other than Raf-1 would involve in the interaction with CaMKII. Indeed, Sst2 somatostatin receptorinhibitscellproliferationthroughRas-,Rap1-, andB-Raf-dependent but not Raf-1-dependent ERK2 activation, causing the p27 induction (34). We are now working on the spatial and temporal pattern of p27 phosphorylation by ERK in vivo.

In summary, we have shown that cross-talk between CaMKII and MAPK signaling, which converges in MEK/ERK activation, leads to the phosphorylation and the degradation of p27, thus promoting cell cycle progression. Our elucidation of the mechanism of the cross-talk between CaMKII and MAPK signaling also has implications for understanding the misregulation of MAPK, p27, and endogenous CaMKII-inhibitory protein hCaMKIINα in diverse human cancers (11, 35) and should foster insights into the intricacies of the CaMKII pathway in modulating cancer cell behavior.

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