Calcium/Calmodulin-Dependent Kinase I and Calcium/Calmodulin-Dependent Kinase Kinase Participate in the Control of Cell Cycle Progression in MCF-7 Human Breast Cancer Cells

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Abstract
Calcium is universally required for cell growth and proliferation. Calmodulin is the main intracellular receptor for calcium. Although calcium and calmodulin are well known to be required for cell cycle regulation, the target pathways for their action remain poorly defined. Potential targets include the calcium/calmodulin-dependent kinases (CaM-K). The aim of this study was to determine the role of the CaM-Ks on cell proliferation and progress through the cell cycle in breast cancer cells. CaM-KI inhibition with either KN-93 or specific interfering RNA (siRNA) caused an arrest in the cell cycle in the human breast cancer cell line, MCF-7. This arrest occurred in the G1 phase of the cell cycle. Supporting this finding, CaM-K inhibition using KN-93 also resulted in a reduction of cyclin D1 protein and pRb phosphorylation when cells were compared with control cultures. Furthermore, inhibition of the upstream activator of CaM-KI, CaM-KK, using siRNA also resulted in cell cycle arrest. In summary, CaM-KK and CaM-KI participate in the control of the G0-G1 restriction check point of the cell cycle in human breast cancer cells. This arrest seems due to an inhibition in cyclin D1 synthesis and a reduction in pRb phosphorylation. To the best of our knowledge, this is the first time that CaM-KK has been reported to be involved in mammalian cell cycle regulation and that CaM-Ks are regulating breast cancer cell cycle.

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Introduction
Breast cancer is the most common malignancy in women. Approximately 1.5 million cases are diagnosed worldwide every year. In the United States, breast cancer is the leading type of cancer and the second leading cause of death by cancer in women (1). In addition to or in conjunction with surgical approaches, systemic therapy (chemotherapy and hormone therapy) and radiotherapy improve the survival of patients with breast cancer. However, these therapies have a number of detrimental side effects. Because of these negative side effects, research efforts have not only focused on chemosensitization and radiosensitization but also in identifying new molecular targets that would allow limited cancer treatment side effects. This is the case of Imatinib (Gleevec) for chronic myeloid leukemia (2) or the recently reported Gefitinib (Iressa) for non–small cell lung cancer (3, 4).

The calcium/calmodulin-dependent kinases (CaM-K) are a family of structurally related serine/threonine protein kinases that include CaM-K kinase (CaM-KK), phosphorylase kinase, myosin light chain kinase (MLCK), and CaM-KI to CaM-KIV (reviewed in ref. 5). CaM-KI, CaM-KII, and CaM-KIV have broad substrate specificity. CaM-KII, phosphorylase kinase, and MLCK have limited substrate specificity. CaM-KK is an upstream activator/kinase for CaM-KI (6) and CaM-KIV (7). The traditional mechanism of activation for the CaM-Ks is through calcium/calmodulin complex binding, which induces phosphorylation of other CaM-Ks (CaM-KI and CaM-IV) by CaM-KK or via autophosphorylation. In certain cell types, oxidative stress can lead to the calcium-independent activation of the CaM-Ks (8, 9). CaM-KI is broadly distributed and is localized in the cytosol. CaM-KIV is mainly expressed in neurons but is also expressed in T cells and testis. CaM-KII is clearly the best characterized of the multifunctional CaM-Ks and is expressed in a variety of tissues (10). Multiple isoforms of CaM-KK and CaM-KII are reported to exist (11, 12).

Cell proliferation can be regulated by growth factors, cytokines, and hormones and is often deregulated in the case of cancer (13). Many signaling pathways are capable of transmitting proliferative signals from the cell membrane or cytoplasm to the nucleus. The role of intracellular calcium has been extensively demonstrated to be required for cell proliferation (reviewed in ref. 14). It is also well known that calcium binds calmodulin and this complex can induce the activation of the CaM-Ks. The CaM-Ks have also been implicated in cell cycle regulation; however, the characterization and magnitude of this involvement has not been well defined. Kahl and Means (14) have noted that a weakness with the majority of the studies describing the role of the CaM-Ks in proliferation is that they have solely involved the use of chemical inhibitors, such as KN-62 or KN-93. These inhibitors are known to suppress CaM-KII activity but have also shown effective in inhibiting CaM-KI and CaM-KIV (5). In a recent article, Kahl and Means showed that CaM-KI is involved in the regulation of the cell cycle at the G1 checkpoint in primary fibroblasts (15). The role of the CaM-Ks in the regulation of the cell cycle in breast cancer cells has not been studied.

We investigated the effect of CaM-K inhibition on cell cycle progression in MCF-7 human breast cancer cells, an extensively used cellular model for breast cancer research. We found that the CaM-K inhibitor KN-93, but not its inactive analogue KN-92, inhibited the proliferation of MCF-7 breast cancer cells. These studies show that arrest occurs primarily in the G1 phase of the cell cycle. Furthermore, we show by silencing specific CaM-K expression with siRNA that CaM-KI and not CaM-KII is responsible for the G1 cell cycle progression in MCF-7 human breast cancer cells. In support of this finding, we found that CaM-KK inhibition with siRNA also resulted in cell cycle arrest.

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Materials and Methods

Cells and cell culture. MCF-7 human breast cancer cells, MCF-10A human breast epithelial cells, and Jurkat T lymphocytes were purchased from American Type Culture Collection (Rockville, MD). MCF-7 cells were grown in complete DMEM supplemented with 10% fetal bovine serum (FBS), 2 mMol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. MCF-10A cells were maintained in complete DMEM/F12 supplemented with 5% FBS, 0.5 μg/mL hydrocortisone, 20 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin, and 10 ng/mL chola rin. Jurkat T cells were grown in complete RPMI 1640 supplemented with 5% FBS, 2 mMol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. All three cell lines were maintained at 37°C with 5% CO2. DMEM, DMEM/F12, and RPMI 1640 were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). FBS was obtained from Atlanta Biologicals (Norcross, GA). EGF, insulin, hydrocortisone, and cholera toxin were purchased from Sigma (St. Louis, MO).

Reagents and antisera. Anti-CaM-KI, anti-CaM-KIV, and anti-CaM-KK antibodies were purchased from BD Biosciences Transduction Labs (San Diego, CA). Anti-CaM-KI and anti-cyclin E antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin D1, anti-cyclin D3, anti-actin, anti-cdk4, anti-cdk6, anti-p27, and anti-pRB antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Anti-actin antibody was purchased from Sigma. Alkaline phosphatase–conjugated goat anti-rabbit IgG (Fc), alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G (H + L), and the 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and nitroblue tetrazolium (NBT) Color Development Substrate (ProteinBlot II AP System) were purchased from Promega (Madison, WI). KN-92 and KN-93 were obtained from Calbiochem (San Diego, CA) and were dissolved in DMSO. LipofectAMINE 2000 and Opti-MEM I were purchased from Invitrogen Life Technologies. All other reagents were purchased from Sigma.

Cell lysis and immunoblot analysis. Cell lysates were prepared as previously described (8). Following harvest, the cells were centrifuged for 1 minute, supernatants were removed, and cell pellets were resuspended in cold lysis buffer [25 mMol/L Tris (pH 7.4), 50 mMol/L sodium chloride, 2% IGEPEAL, 0.2% SDS, and 0.5% sodium deoxycholate] and placed on ice for 15 minutes. Lysates were centrifuged for 10 minutes at 14,000 rpm in a microcentrifuge. Supernatants were removed and placed into fresh tubes. Protein concentrations were calculated using the Bio-Rad Protein Assay (Hercules, CA). Lysates were mixed with 3.3 × sample buffer [200 mMol/L Tris (pH 6.8), 33% glycerol, 66% SDS, 16.6% 2-mercaptoethanol, and 0.094% bromphenol blue]. Samples were boiled for 5 minutes and then frozen at −20°C.

Immunoblotting was done as previously described (16). Twenty micrograms of protein per sample were electrophoresed through SDS-PAGE gels and electrophoretically transferred to polyvinylidene fluoride membranes. Membranes were incubated overnight at 4°C in blocking buffer [50 mMol/L Tris (pH 8.0), 125 mMol/L sodium chloride, 0.1% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide]. Membranes were then incubated for 2 hours with primary antibody diluted in blocking buffer (anti-CaM-KI, 1:1,000; anti-CaM-KII, 1:2,500; anti-CaM-KIV, 1:1,000; anti-CaM-KK, 1:1,000; anti-actin, 1:250; anti-cyclin D1, 1:2,000; anti-cyclin D3, 1:1,000; anti-cyclin E, 1:1,000; anti-cdk4, 1:2,000; anti-cdk6, 1:1,000; anti-pRb, 1:2,000; and anti-p27, 1:1000). The blots were washed twice in TBST [25 mMol/L Tris (pH 8.0), 125 mMol/L sodium chloride, and 0.025% Tween 20] and incubated with an alkaline phosphatase–conjugated goat anti-rabbit immunoglobulin or goat anti-mouse immunoglobulin antibody (Promega; 1:10,000 in TBST) for 1 hour at room temperature. The blots were washed thrice in TBST and developed with the colorogenic substrates BCIP/NBT (Promega ProteinBlot II AP System).

Trypan blue exclusion assay. Cell numbers and viability were evaluated by assessing trypan blue exclusion of cells under light microscopy and scoring the percentage of cells that did not exhibit blue staining as previously described (17). Floating and attached cells were isolated by trypsinization, recovered by centrifugation, and resuspended in complete DMEM. The resuspension was mixed with PBS (1:5) and a serial dilution (1:1) with 0.2% trypan blue solution. Cells were counted using a hemocytometer.

Sulforhodamine B assay. Either MCF-7 or MCF-10A cells in exponential proliferation were trypsinized, counted, and seeded at 6,000 cells in 1 mL of complete DMEM per well in 24-well plates. Optimal seeding densities were determined to ensure exponential growth for 7 days (data not shown). The sulforhodamine B (SRB) assay was done as previously described (18). Briefly, all the culture medium was aspirated and the cells were fixed with cold trichloroacetic acid. Following incubation at 4°C, cells were washed with deionized water. The cells were stained with 0.1% SRB dissolved in 1% acetic acid for 30 minutes and subsequently washed with 1% acetic acid to remove unbound stain. The plates were then left to dry overnight at room temperature. The protein-bound stain was solubilized with 10 mMol/L unbuffered Tris base and transferred to 96-well plates for absorbance readings at 540 nm with background at 690 nm (Anthos Labtech Instruments, Ansthos Reader 2001, Pasadena, CA).

Reverse transcription-PCR analysis of human CaM-KKα and CaM-KKβ mRNAs. One microgram of total RNA per reaction was DNase I treated and converted to cDNA, which was used as a template for PCR. Thirty cycles (95°C for 1 minute, 51°C for 1 minute, and 72°C for 1 minute) were done with the following primers for CaM-KKα sense primer, ACTCACCTTGGAGGAGCCAGTA and antisense primer, GCTG7GAGGACAGCTTTGAAGGT; for CaM-KKβ, we used primers previously described (11). Reverse transcription-PCR (RT-PCR) reactions were done using Ready-To-Go RT-PCR Beads from Amersham Biosciences Co. (Piscataway, NJ).

RNA silencing. SMART pool interfering RNA (siRNA) to target human CaM-KI (Genbank accession no. NM_003656), CaM-KII δ (genbank accession nos. NM_001221, NM_172115, NM_172127, NM_172128, CaM-KII γ (Genbank accession nos. NM_001222, NM_172169, NM_172170, NM_172171, NM_172172, NM_172173), CaM-KK α (Genbank accession nos. NM_032294, NM_172206, NM_172207), and CaM-KK δ (Genbank accession nos. NM_006549, NM_153499, NM_153500, NM_172214, NM_172215, NM_172216, NM_172226) were designed and synthesized by Dharmacon (Lafayette, CO). siRNA (100 mMol/L) was transfected into MCF-7 cells according to the manufacturer’s instructions using LipofectAMINE 2000 in Opti-MEM I. A nonspecific RNA duplex was used in control experiments. Seventy-two hours post-transfection, cells were replated and allowed to grow for 48 hours. Cells were harvested for cell cycle and immunoblot analysis.

Cell cycle analysis by flow cytometry. Cells (0.5-1.0 × 106) were trypsinized, centrifuged, and resuspended in 1 mL PBS buffer (1× PBS and 2% FBS). Three milliliters of ethanol were added and cells were fixed at −20°C for a minimum of 1 hour. Cells were washed and resuspended in 1 mL of PBS buffer. DNA fragmentation assay [200 mMol/L sodium phosphate, dibasic (pH 7.8) and 100 mMol/L citric acid] was added (500 μL) and cells were incubated for 5 minutes at room temperature. Cells were centrifuged and resuspended in 1 mL of propidium iodide (PI) solution (50 μg/mL), 50 μL of RNase A solution (10 mg/mL) was added to each tube, and cells were incubated for 30 minutes. DNA profiles of PI-stained cells were analyzed on a Becton Dickinson FACScan (San Jose, CA) and cell cycle plots generated by data analysis in ModFit LT 3.1 software.

DNA fragmentation assay. The DNA fragmentation assay was done as previously described (19). Cells were washed with 1× PBS, centrifuged, and resuspended in lysis buffer [50 mMol/L Tris-HCl (pH 7.5), 200 mMol/L EDTA, and 1% NP40]. DNA was extracted with isopropanol/ethanol. The degree of fragmentation was analyzed using 3% agarose gel electrophoresis followed by ethidium bromide staining.

Results

The calcium/calmodulin-dependent kinase inhibitor KN-93 reduces proliferation of MCF-7 cells in culture. To determine if inhibition of the CaM-Ks would alter the proliferation of MCF-7 human breast cancer cells, we cultured these cells with the CaM-K inhibitor KN-93. The potential nonspecific effects of KN-93 were assessed with its inactive analogue KN-92. DMSO, the diluent for both KN-93 and KN-92, also served as a control. When compared with untreated cells neither DMSO nor KN-92 altered the number
of cells in these cultures from the time of treatment (day 0) until
day 8. The number of cells increased from 2,600/cm^2 (day 0) to
~50 to 60,000/cm^2 (day 8). However, the CaM-K inhibitor KN-93
significantly reduced cell proliferation in comparison with the
controls (Fig. 1A). When cells were treated with KN-93, the number
of cells in the culture did not increase at all over the 8 days of these
studies. Cell viability was confirmed the same for all experimental
groups by acridine orange/ethidium bromide staining and
observation under fluorescence microscopy (data not shown).
These outcomes show that specific inhibition of the CaM-Ks in
breast cancer cells results in a decrease in cell proliferation and not
in immediate cell death. As can be seen from the photographs of
cells treated with DMSO, KN-92, or KN-93 (Fig. 1B), MCF-7 cells
treated with either DMSO or the inactive analogue KN-92 formed
visually similar colonies of cells by day eight of culture. However,
the cells treated with the CaM-K inhibitor KN-93 failed to establish
colonies 8 days after treatment. These results support our data in
Fig. 1A that demonstrates that inhibition of the CaM-Ks results in a
decrease in the proliferation of MCF-7 breast cancer cells.

**Tumor-forming MCF-7 cells are more susceptible to the**
**antiproliferative effects of KN-93 than the nontumor-forming**
**MCF-10A breast epithelial cell line.** MCF-7 and MCF-10A cells
were cultured 8 days with DMSO, KN-92, or KN-93. Following the
8-day culture, cell proliferation was determined by SRB assay.

When KN-93 (5 μmol/L) was added to the cultures, the
proliferation of MCF-10A cells was 67% in comparison with the
DMSO-treated cells (Fig. 2). When this same concentration was
used on MCF-7 cells, they proliferated only to a level of 40% of the
controls. The apparent discrepancy in proliferation between the
KN-93–treated MCF-7 cells and the same group in Fig. 1A, where
the KN-93–treated cells did not proliferate may be due to technical
differences for proliferation analysis (SRB versus trypan blue assay).

When the KN-93 concentration was increased to 7.5 μmol/L, the
proliferation of the MCF-10A cells was 57%, whereas the MCF-7
cells proliferated to a level of 24% of the controls. These data
indicate the proliferation of tumorigenic MCF-7 cells was more
susceptible to CaM-K inhibition in comparison with nontumori-
genic MCF-10A cells.

**Differential expression of the calcium/calmodulin-dependent**
**kinases in MCF-7 and MCF-10A cells.** Because of the
difference in the susceptibility of MCF-10A and MCF-7 cells to
the down-regulatory effects of CaM-K inhibition, we determined
the expression profiles of the different CaM-Ks in these cell lines.

**Immunoblots (Fig. 3A) showed that CaM-KII y and CaM-KII g**
**isoforms were both expressed in MCF-7 and MCF-10A cells as it
has been previously shown (12). The expression of these two**
**isoforms seemed similar in both cell lines. CaM-KI expression was
detected in MCF-7 cells but not in MCF-10A cells. It is possible that

**Figure 1.** CaM-K inhibitor KN-93 reduces the proliferation of MCF-7 human breast cancer cells in culture. On day –1, MCF-7 cells were
tryptsinized, washed, and seeded at 2,500 cells/cm^2 in 1 mL of
complete DMEM in 24-well plates. A, twenty-four hours later
day 0), the cells were treated with either DMSO (0.1%), KN-92
(5 μmol/L), or KN-93 (5 μmol/L). Cell counts of floating and adherent
cells were done by trypan blue exclusion assay at days 0, 2, 4, 6,
and 8 after the treatments mentioned above. Average of four
independent experiments. The error bars for the KN-93 time points
are not too small to be visible over the squares. B, twenty-four hours
after plating out (day 0), the cells were treated with either DMSO
(0.1%), KN-92 (5 μmol/L), or KN-93 (5 μmol/L). Cells were observed
in an inverted microscope and photographs were obtained at days
0 and 8 after treatment.
PCR analysis was done. Three variants of human CaM-KKa have been identified (Genbank). All three mRNA variants result in primers. MCF-7 cells express both a band observed in immunoblots for MCF-7 cells may be CaM-KKh. CaM-KK band observed in the immunoblots for MCF-10A cells is likely the MCF-7 cells. Furthermore, MCF-10A cells express only the CaM-KKmRNA is being expressed in MCF-10A; however, it must be at a level below detection on our immunoblots. The expression of the CaM-KKs has not been characterized in breast cells. In other cell types, there are two reported isoforms for CaM-KK (CaM-KKα and CaM-KKβ; ref. 20). In addition, some cells express multiple variants of CaM-KKβ (21, 22). In our experiments, the antibody used recognized two bands of CaM-KK in MCF-7 cells and only one band in MCF-10A cells (Fig. 3A). To determine if the two bands seen in MCF-7 cells were either multiple isoforms of CaM-KKβ or represented CaM-KKα and CaM-KKβ, RNA was obtained and RT-PCR analysis was done. Three variants of human CaM-KKα mRNA have been identified (Genbank). All three mRNA variants result in the amplification of a 116-bp fragment using the CaM-KKα primers. MCF-7 cells express both α and β mRNA isoforms of CaM-KK based on the primers used and the sizes of the fragments obtained (Fig. 3B). We do not know the identity of the slightly smaller fragment that is identified using the CaM-KKα primers. It may represent a spliced variant or a yet to be identified isoform. It is known that CaM-KK is alternatively spliced (11). The CaM-KKβ2 isoform was not expressed at readily detectable levels in the MCF-7 cells. Furthermore, MCF-10A cells express only the CaM-KKα mRNA isoform (data not shown), suggesting that the unique band observed in the immunoblots for MCF-10A cells is likely the CaM-KKα isoform. Consequently, the higher molecular weight band observed in immunoblots for MCF-7 cells may be CaM-KKβ2. CaM-KIV was not expressed in either of the breast cell lines but was seen in our positive control (Jurkat). These results would suggest that the specificity of the anti-proliferative effect of KN-93 on MCF-7 breast cancer cells may be due to the inhibition of CaM-KI.

Inhibition of the calcium/calmodulin-dependent kinases with KN-93 reduces cell cycle progression. To further elucidate the anti proliferative effects of the CaM-K inhibitor KN-93, MCF-7, and MCF-10A cells were stained with PI and cell cycle analysis was done by flow cytometry. The cell cycle profile of MCF-7 cells treated with the CaM-K inhibitor KN-93 was characterized by an increase in the percentage of cells in the G0-G1 phase of the cell cycle and a reduction of the fraction of cells in the S phase (Fig. 4A). Following 2 days in culture, 54% and 61% of the cells were found in the G1 peak of DMSO- and KN-92–treated cells, respectively. When cells were incubated for 2 days with KN-93, 75% of the viable cells could be found in the G1 peak. Following 5 days in culture, 62% and 67% of the cells were found in the G1 peak of DMSO- and KN-92–treated cells, respectively. When cells were incubated for 5 days with KN-93, 79% of the viable cells could be found in the G1 peak. In contrast to the number of cells in G1, treatment of cells with KN-93 caused around a 50% reduction in the number of cells in the S phase when compared with the control cultures. These effects were not seen in the cell cycle profile of MCF-10A cells (Fig. 4B), showing the specificity of the CaM-K inhibitor KN-93 for the tumorigenic MCF-7 breast cancer cell line.

The MCF-7 breast cancer cells arrested in the G0–G1 phase of the cell cycle showed an increase in the sub genomic population at 120 hours (up to 20%) suggesting that the growth arrested cells enter the apoptotic pathway. This increase in subgenomic population was not observed at 48 hours suggesting that the KN-93–induced apoptosis is due to the prolonged G0-G1 arrest rather than the toxic effects of KN-93. The increases in the subgenomic population were correlated with DNA fragmentation (Fig. 4C) showing that apoptosis is happening in the KN-93–treated MCF-7 cells. This effect was completely reversed when the cell medium was changed to complete DMEM at 72 hours after treatment (data not shown).
Figure 4. Inhibition of the CaM-Kinases with the CaM-K inhibitor KN-93 prevents cell cycle progression, induces apoptosis, and down-regulates cyclin D1 expression in MCF-7 human breast cancer cells. MCF-7 (A) and MCF-10A (B) cells were trypsinized, washed, resuspended, and seeded at 50,000 cells per well in 2 mL of complete DMEM or complete DMEM/F12 respectively, in 6-well plates. Twenty-four hours later, the cells were treated with either DMSO (0.1%), KN-92 (5 μmol/L), or KN-93 (5 μmol/L). The day of treatment (0 hours), 48 and 120 hours after treatments, cells were trypsinized, washed, and resuspended in complete medium. Cells were stained with PI as previously described. Flow cytometric analysis of the DNA content was done. Analysis of the cell cycle was done using ModFit LT 3.1 for Macintosh. Representation of six independent experiments with similar results.

C and D, MCF-7 cells were trypsinized, washed, resuspended, and seeded at 2 × 10⁶ cells per T75 flask in 15 mL of complete DMEM. Seventy-two hours later, the exponentially growing cells were treated with either DMSO (0.1%), KN-92 (5 μmol/L), or KN-93 (5 μmol/L). C, DNA was precipitated, electrophoretically separated on a 3% agarose gel, visualized by ethidium bromide staining and photographed using the BioRad ChemiDoc XRS system. D, forty-eight and 72 hours after treatment cellular lysates were prepared, protein concentrations were determined, and 40 μg of protein per lane were loaded. Immunoblot analysis was done using antibodies against cyclin D1, cyclin D3, cyclin E, cdk4, cdk6, pRb, p27, and actin. Representation of three independent sets of immunoblots with similar findings.
Calcium/calmodulin-dependent kinase inhibitor KN-93 inhibits cyclin D1 expression. To examine the mechanisms by which KN-93 causes cell cycle arrest in breast cancer cells, exponentially growing MCF-7 cells were treated with KN-93 and cell lysates were obtained at 48 and 72 hours. As observed (Fig. 4D), cyclin D1 protein levels were moderately reduced at 48 hours and disappeared at 72 hours after treatment with KN-93. The levels of cyclin D1 were not affected in the cells treated with DMSO or mildly affected with the negative analogue KN-92. There was a slight decrease in cyclin D3 immunoreactivity in cells treated with KN-93 for 72 hours. A slower migrating band appeared above cyclin D3 in the same treatment group in comparison with controls. It is possible that cyclin D3 is phosphorylated, leading to a decreased cyclin D3 electrophoretic migration. Furthermore, Rb was found in its unphosphorylated form (110 kDa) in cells treated with KN-93. Cells treated with DMSO or KN-92 conserved the phosphorylated form of pRb that is characterized by slower migration (115 kDa). Cyclin E levels did not change in any case. It is possible that the lack of cyclin D1 results in a decrease in pRb that is characteristic of slow migration. Cyclin E levels did not change in any case. It is possible that the lack of cyclin D1 results in decreased levels of cyclin E.

Overall, these results indicate that treatment of breast cancer cells with the CaM-K inhibitor results in decreased levels of cyclin D1. This decrease would result in decreased pRb phosphorylation and the subsequent G0-G1 cell cycle arrest.

**CaM-KI siRNA blocks cell cycle progression in the G0-G1 phase of the cell cycle.** Because it has been shown that KN-93 can inhibit CaM-KI, CaM-KII, and CaM-KIV (5, 23); that MCF-7 cells are more sensitive than MCF-10A cells to the anti-proliferative effects of KN-93; and that MCF-7 cells, but not MCF-10A cells, express detectable levels of CaM-KI, we decided to determine if CaM-KI could be responsible for the disruption of the cell cycle progression. Cells were transfected with specific siRNA directed to silence the expression of CaM-KI, CaM-KII, or CaM-KIIγ. Treatment of the cells with siRNA directed towards CaM-KIIγ resulted in a decrease of CaM-KIIγ protein but not CaM-KK protein (Fig. 5A). Conversely, treatment of the cells with CaM-KI siRNA resulted in a down-regulation of CaM-KIIγ protein but not CaM-KI protein. When cells were treated with both CaM-KI and CaM-KII, siRNA suppression of both proteins could be noted. The suppression of CaM-KIIγ was not complete in this case; however, it should be noted that the cells in all of the experiments were transfected with a total of 100 nmol/L of siRNA (in this case, 50 nmol/L of each siRNA). When the MCF-7 cells were treated with siRNA directed to CaM-KI, suppression in the expression of CaM-KI protein could be noted. Suppression of any nonsilenced CaM-K or actin was not observed when the cells were transfected with any siRNA (Fig. 5A) indicating the specificity of the siRNA.

Five days following the transfection of MCF-7 cells with CaM-KI siRNA an increase in the percentage of cells in the G0-G1 phase of the cell cycle was observed (Fig. 5B). This effect was not observed when the cells were transfected with a negative control siRNA (Fig. 5B). Immunoblot analysis was done using antibodies against CaM-KI, CaM-KII, CaM-KK and actin. A, forty-eight hours later, cells were stained with PI as previously described. Flow cytometry analysis of the DNA content was done. Analysis of the cell cycle was done using ModFit LT 3.1 for Macintosh. Representation of three independent experiments with similar results.
the cell cycle could be noted (Fig. 5B). Furthermore, a reduced fraction of the cells in the S and G2-M phases was evident, when compared with the cells transfected with nonspecific siRNA. In these same experiments, we found that CaM-KI (6) and CaM-KII (6) siRNA did not induce cell cycle arrest. These results suggest that it is CaM-KI and not CaM-KII that controls the G0-G1 phase progression of the cell cycle in MCF-7 human breast cancer cells and suggests a mechanism for the requirement of both calcium and calmodulin in cell proliferation.

Calculated/calmodulin-dependent kinase kinase siRNA causes cell cycle arrest of breast cancer cells in the G1 phase of the cell cycle. CaM-KK has a role in the phosphorylation and activation of CaM-KI (6). MCF-7 cell transfection with siRNA for CaM-KKα or CaM-KKβ isoform down-regulates both bands of immunoreactivity (Fig. 6A). It is possible that the sequences of the siRNAs are targeted to a similar region in CaM-KKα and CaM-KKβ RNA. Regardless, when MCF-7 cells were transfected with siRNA to either CaM-KKα or CaM-KKβ, we observed cell arrest in the G1 phase of the cell cycle when compared with cells either untransfected or transfected with nonspecific siRNA (Fig. 6B). When 50 nmol/L siRNA to CaM-KKβ was used in combination with 50 nmol/L siRNA to CaM-KI, a similar increase in the number of cells in G1 over those seen using siRNA to CaM-KKβ alone was noted. These findings would suggest that CaM-KK is acting via CaM-KI.

Discussion

The aim of this study was to determine if the CaM-Ks were involved in the proliferation and cell cycle regulation of breast cancer cells. By culturing MCF-7 human breast cancer cells in the presence of the CaM-K inhibitor KN-93, or by cell transfection with specific siRNA, we were able to test the effects of CaM-K inhibition on cell cycle progression. Our results indicate that CaM-KK and CaM-KI are required for G1 phase progression in the cell cycle of MCF-7 human breast cancer cells and consequently their proliferation.

In the cell cycle G1 phase, the cells respond to extracellular cues to make the decision whether to replicate DNA and divide or to exit into a resting state (G0; ref. 13). The time point in G1 phase in which this decision is made is called the “restriction point” because after this point the cells are irreversibly committed to complete the cycle (24). This restriction point transition is controlled by CKDs (25, 26). These enzymes contain both regulatory (cyclin) and catalytic (cdk) subunits. Calcium and its intracellular receptor calmodulin are common to all regulatory transitions of the cell cycle (14). Mouse or human cells cultured in medium containing low concentrations of calcium cease cellular division and accumulate in G1 (27). The calmodulin inhibitors W-7 and W-13 prevent proliferation and colony formation in breast cancer cells and other cell lines (28). Traditionally, it has been thought that CaM-KII is controlling the G1 phase restriction point based on results obtained with KN-93 or KN-62. These inhibitors were once thought to be CaM-KII specific (29–31). However, a recent article by Kahl and Means (15) found that overexpression of a kinase-deficient CaM-KI but not a kinase-deficient CaM-KII prevented G1 progression in normal fibroblasts. Although their research was done in nonimmortalized, nontransformed WI-38 fibroblasts and ours in a tumorigenic cell line, our results corroborate that it is CaM-KI and not CaM-KII that is involved in the regulation of the G1 phase of the cell cycle. Furthermore, our study defined that CaM-KK, an upstream activator of CaM-KI, is also participating in the control of the G1 restriction point of the cell cycle of MCF-7 breast cancer cells.

Cyclin D1 function is essential for regulation of the progression through the restriction point from G1 to S phase (32). Cyclin D1
binds cdk4/6, allowing the phosphorylation of pRb. pRb is the restriction point gate keeper as phosphorylated pRb is not able to repress E2F transcriptional activity (13). Consequently, cyclin D1 has been proposed to be a potential target for chemoprevention/treatment of cancer (33). The role of the CaM-Ks with regard to the G1 restriction point seems mediated in part by cyclin D1 accumulation. Cyclin D1 is one of the most commonly overexpressed oncopgenes in breast cancer (34–37). Forty-five percent to 50% of the primary ductal carcinomas overexpress this protein (38, 39). It was recently shown that cyclin D1 is essential for the development of mammary cancers induced by c-neu and v-Ha-ras (40). Cyclin D1 plays a major role in estrogen-induced mitogenesis in breast cancer cells (38, 41-45). Our results suggest that inhibition of CaM-KK and CaM-KI induces a decrease in the levels of cyclin D1; consequently, pRb phosphorylation is reduced. Previous studies have also found a down-regulation of cyclin D1 in NIH-3T3 fibroblasts treated with KN-93 (31). Kahl and Means reported that CaM-KI inhibition causes down-regulation of the activity of the cyclin D1/cdk4 complex but not a reduction in cyclin D1 protein levels (15). In our studies, we observed a decrease in cyclin D1 levels following treatment with KN-93. This may reflect the tumorigenic nature of the cells, as the studies that showed a down-regulation of cyclin D1 by KN-93 used transformed cells, and those that did not used primary cells.

The mechanisms by which the inhibition of CaM-KI causes down-regulation of cyclin D1 protein levels in MCF-7 cells are not clear. Cyclin D1 expression can be regulated either by transcription, translation, or protein stability (32). We observed that cyclin D1 protein levels did not increase in serum-deprived growth-arrested cells released in complete medium in the presence of KN-93 (data not shown). This suggests that CaM-KI may be inducing cyclin D1 transcription and/or translation. CaM-KI is localized in the cytoplasm; however, whether it can translocate to the nucleus is not known (46). If CaM-KI remains in the cytoplasm, it must be regulating a substrate that can translocate to the nucleus and modulate transcription. Very little is known about physiologically relevant substrates for CaM-KI. It has been shown that CaM-KI can cause cyclic AMP–responsive element binding (CREB) protein phosphorylation in vitro. Although there is a CRE sequence on the cyclin D1 promoter, MCF-7 cells do not express detectable CREB (47). Recently, it has been shown that CaM-KI can participate in extracellular signal-regulated kinase (ERK) activation; thus, ERK may be a potential substrate in breast cancer cells (48). Joseph and Means have reported that CaM-KK and CaM-KI in Aspergillus nidulans are involved in the regulation of DNA synthesis (49). Whether CaM-KI is exclusively controlled by CaM-KK for G1-to-S phase transition remains to be elucidated.

The distribution of MCF-7 cells in the different phases of the cell cycle suggests that the cells are not cycling, as time-dependent cell accumulation in G1 phase is not happening. This would imply a block not only in G1 phase but also in S-G2 phases. A potential role was described for CaM-KII in regulating centrosome duplication in Xenopus eggs (50). However, the role of CaM-KII in regulating centrosome duplication in mammalian cells is unknown. In addition, it has been suggested that CaM-KII participates in the G2-M transition in HeLa cells controlling cdc25 phosphorylation (51). Furthermore, CaM-Ks involvement in the S phase is unknown (14). It is possible that when we treat the cells with KN-93, CaM-KI is inhibited, and this leads to a block in the G1 phase. In addition, KN-93 may inhibit CaM-KII and induce a block in G2. However, the percentages of distribution would suggest that the cells are also blocked in the S phase as the number of cells in G2 remains the same. Nevertheless, CaM-KII silencing with siRNA did not cause any apparent cell cycle arrest in S or G2 phase. Another potential explanation is that the lack of cycling cells is an artificial effect because G1-arrested cells are suffering apoptosis as shown. Thus, the distribution would remain similar with no more cell accumulation in G1.

Our laboratory has previously shown that CaM-KII and CaM-KIV can be activated by certain forms of oxidative stress (8, 9). We have observed that CaM-KII can be activated by oxidative stress in MCF-7 breast cancer cells. In the presence of KN-93, MCF-7 cells are more sensitive to the effects of oxidative stress-inducing treatments like radiotherapy and photodynamic therapy. Considering that one compound may inhibit CaM-KI and CaM-KII, causing proliferation arrest in addition to sensitization to oxidative stress-inducing treatments, the idea of treating breast cancer through CaM-K inhibition may be possible.

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References
15. Kahl CR, Means AR. Regulation of cyclin D1/cdk4
30. Tombs BM, Grant S, Westin EH, Krystal G. G1 cell cycle arrest and apoptosis are induced in NIH 3T3 cells by KN-93, an inhibitor of CaMK-II (the multifunctional Ca2+/CaM kinase). Cell Growth Differ 1995;6:1063–70.
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