A Calmodulin-Binding Site on Cyclin E Mediates Ca2+-Sensitive G1/S Transitions in Vascular Smooth Muscle Cells

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Abstract—Calcium transients are known to control several transition points in the eukaryotic cell cycle. For example, we have previously shown that a coordinate elevation in the intracellular free calcium ion concentration is required for G_1- to S-phase cell cycle progression in vascular smooth muscle cells (VSMC). However, the molecular basis for this Ca^{2+} sensitivity was not known. Using buffers with differing $[Ca^{2+}]$, we found that the kinase activity of mouse and human cyclin E/CDK2, but not other G_1/S-associated cell cycle complexes, was responsive to physiological changes in $[Ca^{2+}]$. We next determined that this Ca^{2+}-responsive kinase activity was dependent on a direct interaction between calmodulin (CaM), one of the major Ca^{2+}-signal transducers of eukaryotic cells, and cyclin E. Pharmacological inhibition of CaM abrogated the Ca^{2+} sensitivity of cyclin E/CDK2 and retarded mouse VSMC proliferation by causing G_1 arrest. We next defined the presence of a highly conserved 22 amino acid N-terminal CaM-binding motif in mammalian cyclin E genes (dissociation constant, 1.5 ± 0.1 μmol/L) and showed its essential role in mediating Ca^{2+}-sensitive kinase activity of cyclin E/CDK2. Mutant human cyclin E protein, lacking this CaM-binding motif, was incapable of binding CaM or responding to $[Ca^{2+}]$. Taken together, these findings reveal CaM-dependent cyclin E/CDK2 activity as a mediator of the known Ca^{2+} sensitivity of the G_1/S transition of VSMC. (***Circ Res*****. 2006;98:1273-1281.)**

Key Words: calcium ■ calmodulin ■ cell cycle ■ cell cycle progression ■ CDK2 ■ cyclin E ■ vascular smooth muscle cells

Vascular smooth muscle cells (VSMC) are the end-effector organs in vasculoproliferative diseases such as atherosclerosis, restenosis, and hypertension.[1] Elucidating the molecular mechanisms underlying the growth of VSMC may help to design novel therapies aimed at preventing and treating these conditions.

Many studies have implicated Ca^{2+} as a regulator of distinct "checkpoints" in the cell cycle of eukaryotes.[2] Mammalian cells appear most sensitive to the depletion of extracellular Ca^{2+} at 2 points, in early G_1 and near the G_1/S boundary.[3] In several cell types, DNA synthesis could be completely inhibited when extracellular Ca^{2+} was chelated by EGTA or when the influx of extracellular Ca^{2+} was blocked by cobalt.[4,5] Specifically for VSMC, it has been reported that depletion of Ca^{2+} stores in the G_1 phase of VSMC results in a profound G_1 arrest that is not overcome until internal Ca^{2+} stores are replenished.[6,7] We also found, in cultured VSMC, that intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) exhibit significant increases as a function of cell cycle, such that resting free $[Ca^{2+}]_i$ vary from $\approx$75 nmol/L at G_0 to $\approx$150 nmol/L at G_1/S, whereas sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA)-maintained releasable $[Ca^{2+}]_i$ vary from $\approx$100 nmol/L at G_0 to $\approx$500 nmol/L at G_1/S.[8–10] Importantly, preventing increases in resting and releasable $[Ca^{2+}]_i$ inhibited G_1- to S-phase transitions.[8,9] Similarly, SERCA inactivation by thapsigargin has been shown to lengthen the G_1 phase of HEK 293 cells.[11]

Calmodulin (CaM), a small acidic protein of 16.7 kDa, is an important transducer of Ca^{2+} signals in eukaryotic cells. It is recruited by a large number of proteins, collectively termed CaM-binding proteins (CaMBPs), through which Ca^{2+} sensitivity is expressed in a variety of cell biological functions.[12] The role of CaM as a regulator of cell cycle progression has also been well established.[2] Early indications came from experiments in which the mitotic cycle was arrested by anti-CaM drugs added to proliferating cells or to cells reentering cell cycle under the influence of mitogens.[13] In addition, it was reported that monoclonal antibodies against CaM inhibited the synthesis of DNA in permeabilized cells.[14] Moreover, progression through G_1 and mitosis exit was observed to be sensitive to changes in the intracellular concentration of CaM.[15] Although others have suggested a

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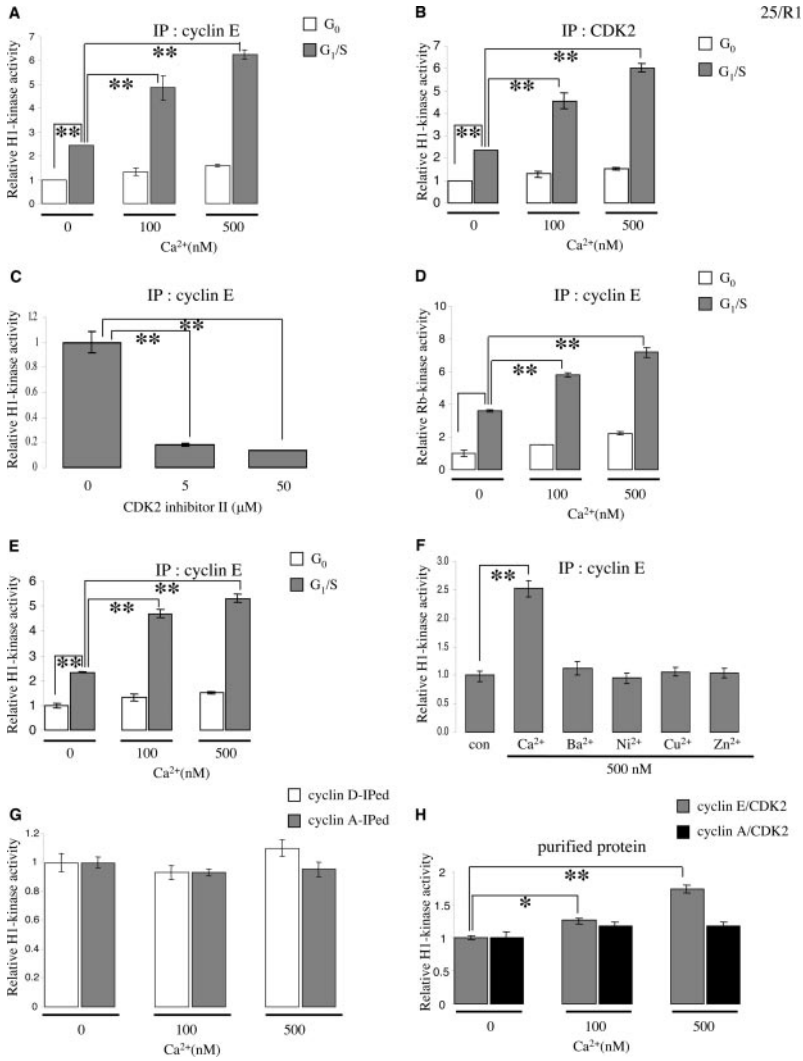

Figure 1. Ca^{2+}-sensitive cyclin E/CDK2 activity in G_1/S-synchronized VSMC. A through C, Histone–H1 kinase activities of cyclin E–IPd (A) and CDK2–IPd (B) complexes from G_0- and G_1/S-synchronized MOVAS in 0 to 500 nmol/L [Ca^{2+}] and with CDK2 inhibitor II in G_1/S extracts at 500 nmol/L [Ca^{2+}] (C). D, Rb kinase activities in MOVAS. E, Cyclin E–IPd complexes from G_1/S-synchronized primary mouse aortic VSMC. F, Effects of 0 (con) or 500 nmol/L divalent cations on cyclin E–IPd complexes from G_1/S-synchronized MOVAS. G, Cyclin D–IPd complexes from early G_1 MOVAS (10% FBS for 10 hours) and cyclin A–IPd complexes from S-synchronized MOVAS (10% FBS for 20 hours). H, Purified cyclin E/CDK2 and cyclin A/CDK2 in the presence of 500 ng of purified CaM. All experiments were performed in triplicate on at least 2 separate occasions and have been normalized to the kinase activity at 0 (or 500) nmol/L [Ca^{2+}] at G_0 (or G_1/S, as appropriate). *$P<0.05$, **$P<0.01$.

role for CaM in the G_1/S transition through the activation or expression of proteins involved in DNA replication,[16,17] and the phosphorylation of the retinoblastoma protein (Rb),[18] the molecular and cell biological mechanisms through which Ca^{2+} and or CaM exerted these effects remained obscure.

Thus, the primary objectives of our study were to identify which cell cycle proteins expressed in VSMC were responsive to changes in [Ca^{2+}], and whether their Ca^{2+} sensitivity was mediated by CaM, and how. Given that (1) cyclin E/CDK2 is an enzyme complex that is both rate limiting and essential for S phase entry[19,20] and (2) cyclin E synthesis begins in G_1 and reaches a peak at the G_1/S transition,[21,22] we hypothesized that the cyclin E/CDK2 complex may mediate Ca^{2+} sensitivity of the G_1/S cell cycle transition of VSMC. The current study reports that this is the case and that specific CaM-cyclin E interactions underlie this.

Materials and Methods

Materials and Cell Culture

Purified active cyclin E/CDK2 and cyclin A/CDK2 were obtained from Upstate Biotechnology (Lake Placid, NY). CDK2 inhibitor II, KN-93, and cyclosporin A were obtained from Calbiochem (La Jolla, Calif). Calmidazolium, dithiothreitol (DTT), aprotinin, leupeptin, and other chemicals were purchased from Sigma (St Louis, Mo). Synthetic peptides were purchased from GenScript Corp (Piscataway, NJ).

The isolation and culture of primary mouse aortic VSMC and the characterization of MOVAS, a mouse VSMC line, have been described.[10] MOVAS were grown in DMEM (GIBCO/BRL, Gaithersburg, Md) supplemented with 10% FBS (Hyclone, Logan, Utah) and 1% penicillin-streptomycin (GIBCO/BRL). For serum starvation, cells were grown to 60% to 70% confluence, washed twice with PBS, and cultured in starvation medium (DMEM without FBS) for 48 hours to achieve G_0 arrest. Fresh DMEM with 10% FBS was used to initiate reentry into cell cycle, and cells were incubated for 16 hours to allow them to approach the G_1/S transition or for 20 hours for S-phase synchronization. Primary mouse aortic SMC were grown in media supplemented with platelet-derived growth factor (PDGF) (50 ng/mL). G_0 synchronization in these cells was achieved with 72 hours of 0.25% FBS-supplemented DMEM. Progression to G_1/S was brought about by stimulation with 10% FBS and 50 ng/mL PDGF for 24 hours.

Immunoprecipitation and Kinase Assays

SMC (5×10^6) were harvested and resuspended with 2 mL of lysis buffer (50 mmol/L Tris [pH 7.4], 250 mmol/L NaCl, 5 mmol/L EDTA, 0.1% NP-40, 0.5 mmol/L DTT, 0.1 mmol/L Na_3VO_4, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 2 mmol/L PMSF, and 10% glycerol). After homogenization, cell lysates were clarified by centrifugation at 12×10^3 rpm, 4°C for 30 minutes, and the superna-

tant was collected. An aliquot was taken for protein concentration determination using the BCA protein assay kit (Sigma). Cell extracts (200 μg) were then incubated for 2 hours at 4°C with saturating concentrations of polyclonal antibodies (Abs). Immune complexes were collected by incubation with GammaBind G Sepharose resin (Amersham Pharmacia, Piscataway, NJ) for 1 hour at 4°C. The beads were then washed three times with washing buffer (50 mmol/L Tris [pH 7.4] and 1 mmol/L DTT).

For the in vitro kinase assays, 20 μL of kinase reaction mixture (20 mmol/L Tris [pH 7.4], 5 mmol/L $MgCl_2$, 2.5 mmol/L $MnCl_2$, 1 mmol/L DTT, 10 μg of C terminal of human Rb protein or 4 μg of histone–H1 [both Upstate Biotechnology] as substrates, 20 μmol/L ATP, and 2.4 μCi [γ- ^{32}P]ATP [Amersham Pharmacia]) was added to CDK2-, cyclin E–, or cyclin A–immunoprecipitated (IPd) complexes after removing the washing buffer from GammaBind G Sepharose resin. Based on the total calcium content of kinase reaction mixtures (as determined by inductively coupled plasma atomic emission spectrometry), amounts of EGTA required to yield final concentrations of free Ca^{2+} ion (0, 100 or 500 nmol/L) were calculated by Winmol/LaxC (http://www.stanford.edu/~cpatton/winmaxc2.html). After 30 minutes of incubation at 37°C, reactions were stopped by adding 20 μL of 2× sodium dodecyl sulfate (SDS) loading buffer and heating at 100°C for 5 minutes. Labeled proteins were resolved by 16% SDS-PAGE. Phosphorylated Rb or histone–H1 bands were visualized by autoradiography and quantified in a Scintillation Counter LS6500 (Beckman Coulter, Fullerton, Calif) after being cut from the gel.

Western Blot

Between 20 to 30 μg of cell extracts were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes (Sigma, 0.2 μmol/L pore size). Blots were blocked with 5% nonfat dry milk in TBS-T (10 mmol/L Tris [pH 8.0], 150 mmol/L NaCl, and 0.05% Tween-20) overnight at 4°C and then incubated for 3 hours at 25°C in TBS-T plus 3% nonfat dry milk containing primary Abs, including anti-cyclin E and -cyclin A (Upstate Biotechnology), -phospho-CDK2 at Thr^{196} (Cell Signaling Technology, Danvers, MA), -CaM, -His probe, and -actin (Santa Cruz Biotechnology, Santa Cruz, Calif). Protein bands were detected with ECL Reagents (Amersham Pharmacia), with horseradish peroxidase–conjugated secondary goat anti-rabbit IgG (Santa Cruz Biotechnology). Quantification of band intensity was performed using Quantity One (Bio-Rad, Hercules, Calif).

CaM-Binding Analysis

Purified cyclin E/CDK2 and cyclin A/CDK2 (100 ng) (Upstate Biotechnology) complexes were diluted with Ca^{2+}-binding buffer (50 mmol/L Tris [pH 7.5], 100 mmol/L NaCl, and 2 mmol/L $CaCl_2$) into a final 20 μL and incubated with an equal volume of preequilibrated CaM–Sepharose 4B resin (Amersham Pharmacia) for 2 hours at 4°C. After centrifugation, the supernatant containing unbound proteins was removed and the resin was washed twice with the buffer used in the binding step. After removing buffer completely from the resin, SDS loading buffer with DTT was added to the dried resin to collect all proteins bound to CaM. The proteins were resolved by 12% SDS-PAGE and visualized by silver staining to determine the amount bound to resin-immobilized CaM.

Confocal Immunofluorescence

Methods and representative images used for analysis of the extent of CaM and cyclin E colocalization are shown in the online data supplement available at http://circres.ahajournals.org.

Flow Cytometric Analysis

Samples were prepared as previously described with minor modifications.[8] Cells were counted in a flow cytometer (FACScan; BD Biosciences, Franklin Lakes, NJ), and G_0/G_1, S, and G_2/M cell percentages were calculated with Cell Quest software (BD Biosciences).

Fluorescence Titration

The methods used to determine the dissociation constant (K_d) for peptide–protein binding are detailed in the online data supplement.

Transfection

pIRESpuro3 plasmid vectors (Clontech, Palo Alto, Calif) harboring wild-type (wt) and N-terminal–deleted mutant (Nδ) cyclin E were obtained by PCR amplification of a pECE human cyclin E plasmid (a generous gift of Dr Paul Hamel, University of Toronto) with oligonucleotides 5′-ccggaattcatgaaggaggacggcg-3′ (forward wt cyclin E), 5′-ccggaattcatgccagatgaagaaatgg-3′ (forward Nδ cyclin E), and 5′-ccggaattctcagtggtggtggtggtggtgcgccatttccggccc-3′ (reverse for both). DNA sequences of each construct were confirmed by sequencing. Transfections were performed with the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, Calif) according to the instructions of the manufacturer. Starting 48 hours posttransfection, MOVAS were cultured in the presence of 5 μg/mL puromycin for 2 weeks.

Statistics

ANOVA, Student *t* test, and coefficients of variance tests were used as appropriate. Analyses were performed on SPSS version 13.0 (Chicago, Ill).

Results

Cyclin E/CDK2 Activity at the G_1/S Transition of VSMC Is Ca^{2+} Sensitive

Kinase activities of cyclin E–IPd complexes from G_0- and G_1/S-synchronized MOVAS were analyzed in different [Ca^{2+}]. We observed, at 0 nmol/L [Ca^{2+}], that histone–H1 kinase activity at G_1/S was greater than at G_0 (2.4-fold; Figure 1A). These data suggested a G_1/S-associated increase in cyclin E/CDK2 activity, which has been reported previously.[23] Our first novel finding was that an increase in reaction buffer [Ca^{2+}] from 0 to 500 nmol/L consistently increased histone–H1 kinase–defined activity of cyclin E–IPd complexes from G_1/S- (2.6-fold) but not G_0-synchronized VSMC (Figure 1A). Identical results were obtained when the experiment was performed using CDK2-IPd complexes (G_1/S: 2.7-fold; Figure 1B). Experiments with CDK2 inhibitor II showed that the histone–H1 kinase–defined activity of cyclin E immunoprecipitates (IPs) was CDK2-dependent (Figure 1C). Of note, Rb kinase-defined activity of cyclin E IPs was also Ca^{2+} responsive only at G_1/S (Figure 1D). In addition, histone–H1 kinase activity of cyclin E–IPd complexes from primary mouse aortic VSMC exhibited the same Ca^{2+} responsiveness as those from MOVAS (G_1/S: 2.3-fold; Figure 1E). As a next step, we determined that this enhancement was specific to an increased [Ca^{2+}] and not other divalent cations (Figure 1F).

Unlike cyclin E/CDK2, cyclin D/CDK4 and cyclin A/CDK2 exhibited different properties. Neither cyclin D–IPd complexes from early G_1 phase MOVAS nor cyclin A–IPd complexes from S-phase MOVAS (Figure 1G) showed Ca^{2+} responsive increases in histone–H1 kinase activity. In addition, purified cyclin A/CDK2 complexes did not exhibit the Ca^{2+} sensitivity observed with purified cyclin E/CDK2 (Figure 1H). Of interest, the ability of Ca^{2+} to enhance histone–H1 kinase activity of purified cyclin E/CDK2 complexes (1.7-fold; Figure 1H) was less than that observed in IPs of cell extracts (>2.5-fold; Figure 1A through 1F), suggesting perhaps that factors other than those present in "purified" complexes participate in their Ca^{2+} sensitivity.

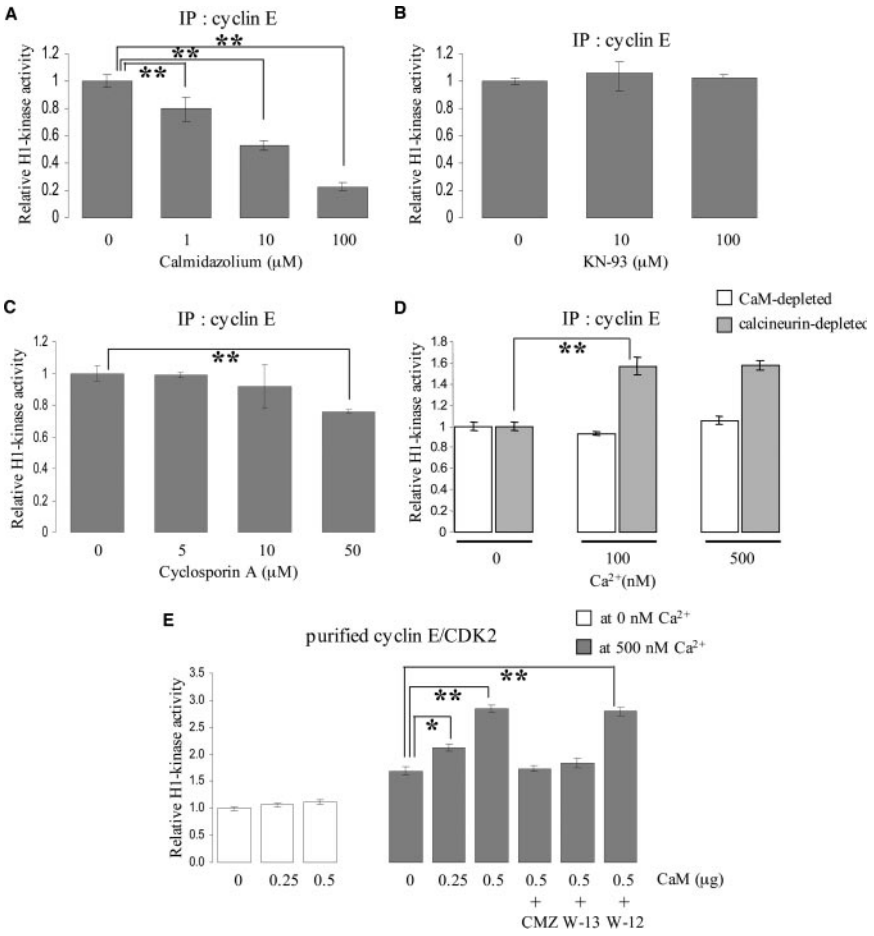

Figure 2. CaM-dependent Ca^{2+} sensitivity of cyclin E/CDK2. H1 kinase activities of cyclin E–IPd complexes from G_1/S-synchronized MOVAS at 500 nmol/L $[Ca^{2+}]$ with calmidazolium (A), an inhibitor of CaM; KN-93 (B), an inhibitor of CaMKs; cyclosporin A (C), an inhibitor of calcineurin; and following CaM or calcineurin immunodepletion (D). E, Effects of increasing purified [CaM] on purified cyclin E/CDK2 at 0 and at 500 nmol/L $[Ca^{2+}]$. Calmidazolium (CMZ), W-13, and W-12 (all 100 μmol/L) were added to examine whether the enhancement of cyclin E/CDK2 activity was CaM dependent. All experiments were performed in triplicate on at least 2 separate occasions and have been normalized to the kinase activity at 0 drug or $[Ca^{2+}]$. $^*P<0.05$, $^{**}P<0.01$.

Taken together, the above data showed that the activity of cyclin E/CDK2 on 2 important substrates (Rb and histone–H1) involved in the G_1/S transition is Ca^{2+} sensitive within the physiological range of $[Ca^{2+}]_i$ known to accompany this stage of the cell cycle in VSMC.[8–10] These data implicated cyclin E/CDK2 as a potential mediator of the known Ca^{2+} sensitivity of the G_1/S transition of both primary mouse VSMC and the MOVAS cell line.

CaM and, to a Limited Extent, Calcineurin, but Not CaM-Activated Kinases, Transduce Ca^{2+}-Sensitive Cyclin E/CDK2 Activity in VSMC

We next explored what mediated the Ca^{2+} sensitivity of cyclin E/CDK2. A potential role for CaM, among the major Ca^{2+} signal–transducing proteins in eukaryotes, was examined first. When added to cell extracts, the potent CaM inhibitor calmidazolium inhibited cyclin E/CDK2 activity in a dose-dependent manner (Figure 2A), suggesting a role for CaM in regulating cyclin E/CDK2 activity. As known downstream mediators of CaM-regulated cell cycle progression in various cell types, the potential roles of multifunctional CaM-activated kinases (CaMKs) and calcineurin were tested next. KN-93, an inhibitor of several CaMKs,[24] did not affect H1 kinase–defined cyclin E/CDK2 activity (Figure 2B). Only high concentrations of cyclosporin A (50 μmol/L), an inhibitor of calcineurin,[25] exhibited at most 20% inhibition of cyclin E/CDK2 activity (Figure 2C).

To test directly whether CaM and calcineurin are necessary for Ca^{2+} sensitivity of cyclin E/CDK2 activity, immunodepletion experiments were performed. To deplete CaM or calcineurin from cell extracts, immunoprecipitation (IP) was first performed with excess amounts of anti-CaM or anti-calcineurin Abs, followed by a second IP with anti-cyclin E Ab on CaM- or calcineurin-depleted cell extracts. CaM-depleted cell extracts did not exhibit any Ca^{2+}-mediated stimulation of cyclin E/CDK2 activity, suggesting that CaM is essential for Ca^{2+} sensitivity of cyclin E/CDK2 activity (Figure 2D). By contrast, calcineurin-depleted cell extracts still exhibited some Ca^{2+} sensitivity (Figure 2D).

To determine whether CaM has a direct effect on cyclin E/CDK2 activity, purified CaM (Sigma; purity >95%) was titrated with a fixed amount of purified human cyclin E/CDK2. CaM increased cyclin E/CDK2 activity in a dose-dependent manner only in the presence of Ca^{2+}, an effect that was abolished by calmidazolium and W-13 (another CaM antagonist) (Figure 2E), suggesting that CaM has a direct stimulatory effect on cyclin E/CDK2 activity. W-12, an analog of W-13 with much lower CaM affinity, did not mimic the effect of W-13 (Figure 2E). Together, the above data suggested that the stimulatory effect of CaM on cyclin E/CDK2 activity was mediated primarily by a direct Ca^{2+}-dependent mechanism involving CaM and cyclin E/CDK2, rather than through CaMKs or calcineurin.

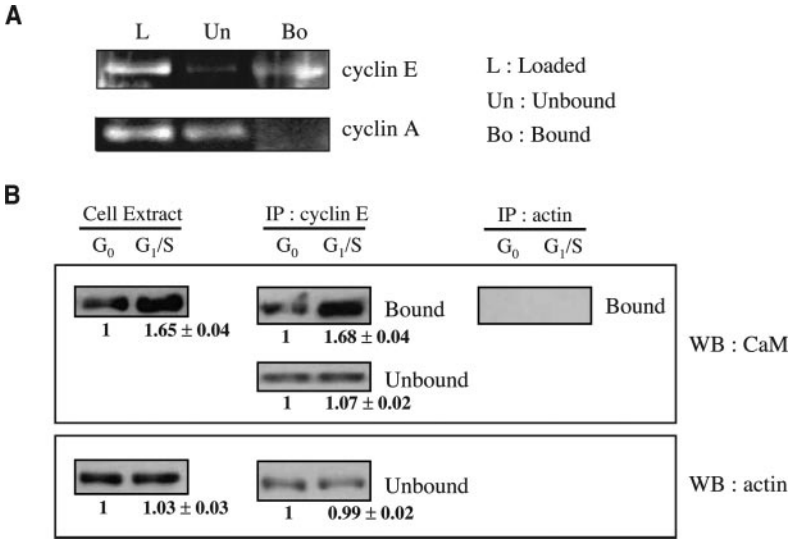

Figure 3. Physical interaction between CaM and cyclin E. A, Silver staining for CaM–Sepharose 4B–bound cyclin E and cyclin A. Purified cyclin E/CDK2 and cyclin A/CDK2 (100 ng) were diluted in Ca^{2+}-binding buffer and applied to a CaM–Sepharose 4B resin. Unbound proteins were collected (Un), and bound proteins (Bo) were eluted with SDS buffer after several washes. An identical amount of each protein loaded on the binding column was also electrophoresed (L). B, Western blot analysis of CaM in whole cell extracts and in cyclin E–IPd extracts from G_0- and G_1/S-synchronized primary mouse VSMC. Actin staining established protein loading. Representative blots from 3 separate experiments are shown with normalized band intensities. $^{**}P<0.01$ vs G_0.

Interaction Between CaM and Cyclin E Is Essential for Ca^{2+}-Sensitive Kinase Activity of Cyclin E/CDK2

To explore further the mechanisms underlying CaM-dependent Ca^{2+}-sensitive cell cycle kinase activities in VSMC, we used a CaM-binding assay. Equal amounts of purified cyclin E/CDK2 and cyclin A/CDK2 were loaded on a CaM–Sepharose column, and silver-stained blots of bound and unbound fractions revealed binding between CaM and cyclin E but not between CaM and cyclin A (Figure 3A). With varying buffer [Ca^{2+}], these experiments revealed that the interaction between CaM and cyclin E occurs in a Ca^{2+}-dependent manner (data not shown). These results were entirely consistent with the observed difference in Ca^{2+}-sensitivities between cyclin E/CDK2 and cyclin A/CDK2 (Figure 1H). Moreover, these data further supported a direct interaction between CaM and cyclin E, not CDK2, as an essential requirement for Ca^{2+} sensitivity of cyclin E/CDK2.

Co-IP analysis between CaM and cyclin E revealed that the amount of CaM bound to cyclin E increased at G_1/S (Figure 3B; 1.68-fold). The absence of any CaM in IPs that used a nonspecific Ab (anti-actin) supported further the specificity of the interaction between CaM and cyclin E. In addition, subcellular localizations of CaM and cyclin E were visualized in MOVAS by fluorescence confocal microscopy, and the proportion of cells showing strong colocalization signals were calculated in both G_0- and G_1/S- synchronized populations (n=1000 cells each). This analysis suggested a 62% increase in the number of nuclei with strong CaM and cyclin E colocalization at G_1/S versus G_0 (378 versus 233; $P<0.001$; online data supplement), a result mirroring closely the observed increase (68%) in co-IP experiments (Figure 3B). This may be related to increased expression levels of CaM at G_1/S (Figure 3B), a finding also reported by others,[13,26] and/or the higher $[Ca^{2+}]_i$ of G_1/S-, as compared with G_0-synchronized cells.[8–10]

Inhibition of CaM Retards the Proliferation of VSMC

Having demonstrated a putative molecular mechanism mediating Ca^{2+}-sensitive G_1/S transitions, we sought to directly test its physiological significance. Flow-assisted cell sorting analysis (FACS) of DNA content was performed to determine whether inhibition of CaM retards the cell cycle progression of VSMC. Whereas control experiments showed that 24.4% of primary mouse aortic SMC had entered S phase after 24 hours of serum stimulation, only 10.8% of cells treated with calmidazolium, and 12.6% of cells treated with W-13, had done so ($P<0.001$; Figure 4A). A similar level of cell cycle inhibition was also observed in MOVAS (online data supplement).

Western blot analysis in primary VSMC showed that the interaction between CaM and cyclin E was severely inhibited by calmidazolium (Figure 4B, lanes 5 and 6), whereas the amounts of cyclin E and CaM itself were not (Figure 4B, lanes 2 and 3). This result indicated that the observed decrease in cyclin E-CaM interaction is not simply attributable to the inhibition of cell cycle progression. Importantly, the level of Thr160-phosphorylated CDK2 (the active form of CDK2) was inhibited by calmidazolium treatment (Figure 4B, lanes 2 and 3).

Together, these data suggested (1) that CaM facilitates G_1- to S-phase transitions in VSMC via binding to cyclin E and (2) that this interaction may play a role in activating CDK2.

Identification of the CaM-Binding Motif on Cyclin E

To provide a structural basis for our functional model, we next sought to identify the CaM-binding region on cyclin E. A CaM-target database (http://calcium.uhnres.utoronto.ca/ctdb)[27] predicted a CaM-binding motif on human cyclin E with successively high probability scores (Figure 5A). This consisted of 22 amino acids near the N terminus of human cyclin E (amino acids 5 to 26), with 5 hydrophobic and 5 basic residues predicted to be critical for a hydrophobic interaction between CaM and cyclin E. We termed this motif the "calmodulin-binding sequence" (CBS) of cyclin E and noted that mouse and rat cyclin E genes also harbor this conserved sequence, whereas the *Drosophila* and *Caenorhabditis elegans* homologs do not (Figure 5B), a finding that may restrict the physiological relevance of this domain to mammals. Consistent with the results of our kinase activity assays,

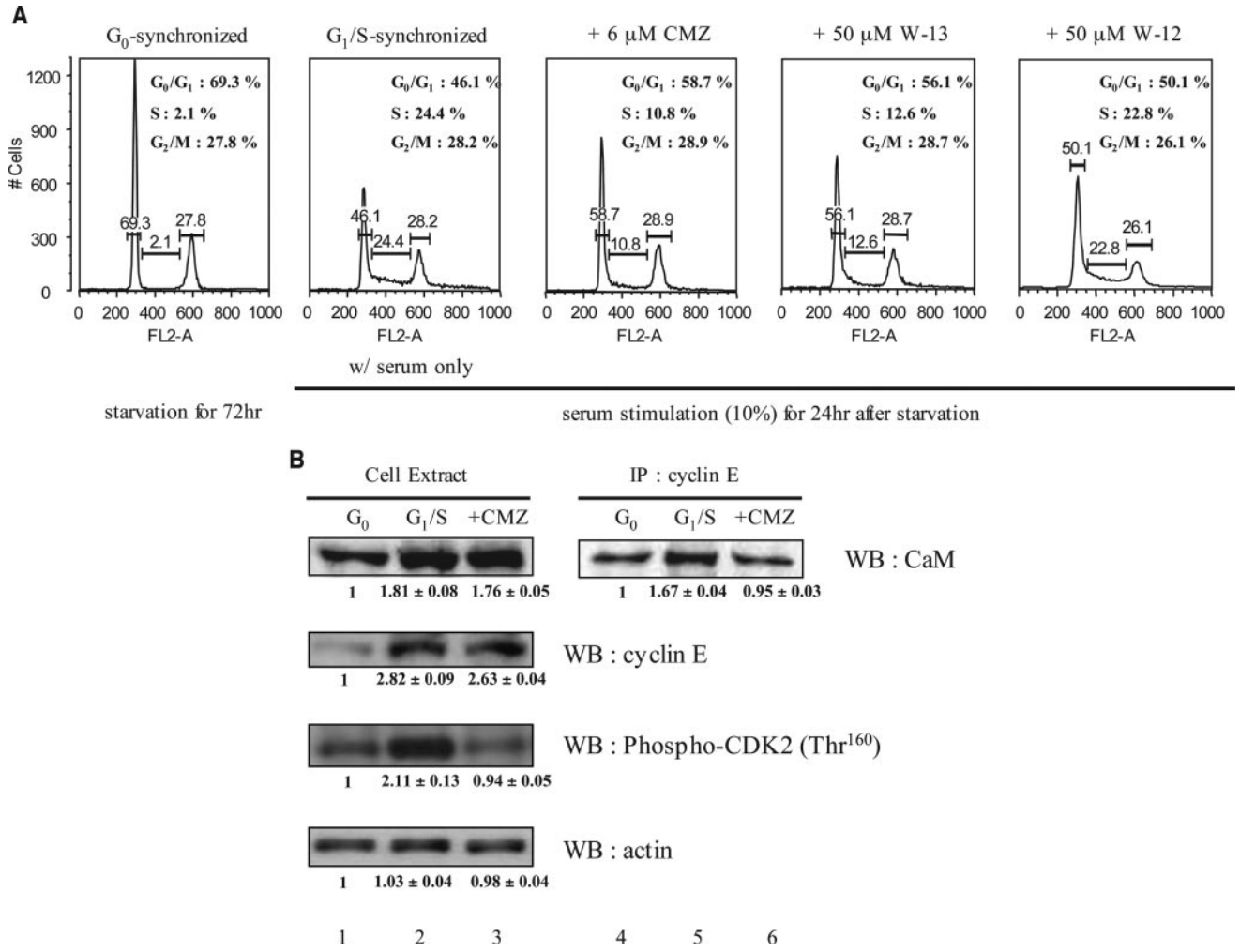

Figure 4. CaM-responsive cell cycle progression in VSMC. A, Flow cytometric analysis of DNA content in G_0- and G_1/S-synchronized, calmidazolium- (CMZ) (6 μmol/L), or W-13– or W-12–treated (both 50 μmol/L) primary VSMC. Please note that CMZ-, W-13–, or W-12–treated primary VSMC were also serum stimulated for 24 hours. B, Western blot analysis for CaM, cyclin E, and CDK2 (phosphorylated at Thr160) in whole cell extracts and cyclin E–IPd extracts (only for CaM) from G_0- and G_1/S-synchronized and CMZ-treated primary VSMC. Representative blots from 3 separate experiments are shown with normalized band intensities. $^{**}P<0.01$ vs G_0.

no similar CBS was revealed in alignment and sequence analyses of all cyclins D and A.

To confirm the ability of the CBS of cyclin E to bind CaM, we performed tyrosine residue–based fluorescence titration experiments using purified peptides in vitro. Figure 5C shows a representative binding profile of the CBS peptide to CaM. The observed decrease in the relative intensity indicates changes in the environment of any (or all) of the 3 tyrosine residues (2 in CaM and 1 in the CBS peptide) associated with the binding event. The fit of the fluorescence profile (see online data supplement for details) yielded a CBS-CaM dissociation constant (K_d, $1.5\pm0.1\times10^{-6}$ mol/L) consistent with other CaM-binding proteins such as caldesmon (K_d, 0.23×10^{-6} mol/L) and calponin (K_d, 1.3×10^{-6} mol/L).[28]

Nδ Cyclin E Neither Binds CaM nor Exhibits Ca^{2+} Sensitivity

To investigate further the structural basis and functional importance of CaM–cyclin E interactions, expression vectors harboring either His-tagged full length (wt) or His-tagged Nδ mutant forms of human cyclin E (lacking amino acids 1 to 26) were stably transfected into MOVAS (Figure 6A). Despite equal levels of His-tagged wt and Nδ cyclin E protein expression (Figure 6B, left panel), co-IP analyses on G_1/S-synchronized cell populations indicated that only wt cyclin E could bind CaM (Figure 6B, right panel). Moreover, histone–H1 kinase assays performed on G_1/S-synchronized cells revealed that despite an equivalent level of activity at 0 nmol/L [Ca^{2+}] to wt cyclin E/CDK2, Nδ cyclin E/CDK2 complexes did not exhibit any appreciable Ca^{2+}-sensitive enhancement of function. By contrast, IPd wt human cyclin E exhibited an identical level of Ca^{2+} sensitivity as endogenous mouse cyclin E in vector only transfected cells (2.5-fold; Figure 6C).

Discussion

This study was focused on identifying the molecular mechanism(s) underlying the poorly understood Ca^{2+} sensitivity of the G_1 to S-phase cell cycle transition of VSMC. The results presented here implicate cyclin E/CDK2 in this process and show that a Ca^{2+}-dependent binding of CaM to cyclin E at

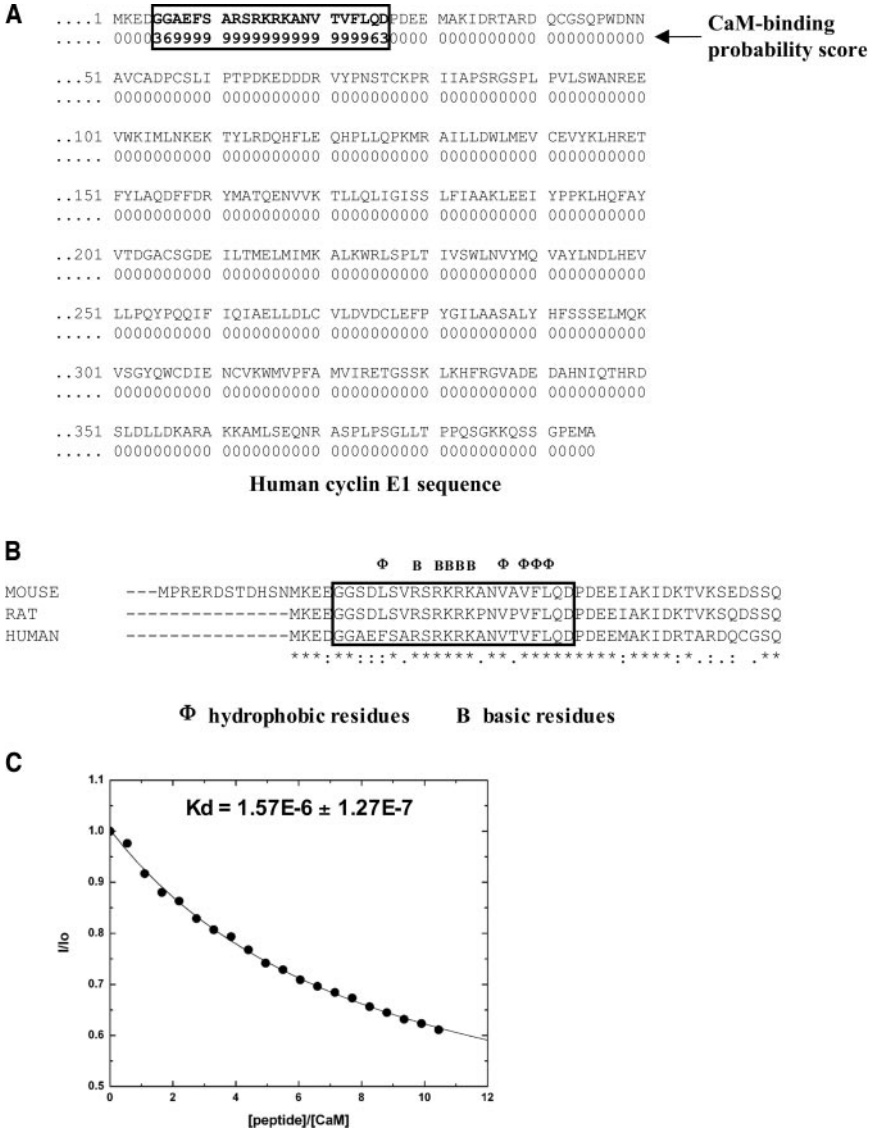

Figure 5. Identification of a CaM-binding motif on cyclin E. A, The putative CBS on human cyclin E as predicted by a CaM-target database is shown. B, The N-terminal sequences of mouse, rat, and human cyclin E all harbor this conserved putative CaM-binding site. C, Representative CaM-CBS peptide fluorescence titration (ie, binding) profile and K_d were calculated as described in Materials and Methods.

G_1/S is critical for G_1/S- and Ca^{2+}-specific enhancement of cyclin E/CDK2 activity. We also defined a CaM-binding motif in the human cyclin E gene and demonstrated its essential role in mediating Ca^{2+}-sensitive CDK2 activity.

Although the expression of CDK2 in T lymphocytes was noted to be depressed by the CaM antagonist W-13,[29] our work is the first to show that CaM increases CDK2 activity via binding to cyclin E. This conclusion was supported by various analyses using several independent approaches including Western blot, co-IP, immunohistochemistry, peptide binding, and structure–function studies with mutants. To investigate further, fluorescence spectroscopy was used to calculate K_d between the CBS peptide and CaM. The finding that Nδ cyclin E was unable to bind to CaM (Figure 6B) strongly suggested that this CaM-binding motif in human cyclin E1 (amino acids 5 to 26) is the only area involved in CaM–cyclin E interactions. As such, the determined K_d obtained from CaM and the CBS peptide likely reflects the true dissociation constant between CaM and cyclin E. If the fluorescent residues examined were far from the binding site or were not involved in a binding-mediated conformational change, no changes in fluorescence would have been observed.[30] Rather, our experiments clearly support a structural relationship between the CBS and CaM with a K_d consistent with other CaMBPs such as G protein–coupled receptor kinase 2,[31] caldesmon, calponin,[28] spectrin,[32] and CaM-dependent adenylate cyclases.[33,34]

Although previous reports have suggested a role for CaM in cell cycle,[2] specific molecular pathways through which CaM might exert these effects were not known. Kahl and Means implicated CaM in the regulation of cyclin D1/CDK4 through their discovery that CaMKI, and not CaMKII, was the KN-93–sensitive CaMK responsible for KN-93–induced G_1 arrest in WI-38 fibroblasts.[35] They reported also that CaM activates cyclin D1 protein synthesis in fibroblasts via calcineurin.[36] Previously, Taules and colleagues had implicated CaM in the nuclear entry of cyclin D/CDK4 and identified a relevant interaction between CaM and the CDK4 inhibitor $p21^{Cip1}$.[37,38] In contrast to the work in other cell types, we did not find KN-93–sensitive CaMKs to be involved in mediating Ca^{2+}/CaM sensitivity of cyclin E/CDK2 complexes and demonstrated a very modest effect of cyclosporin A and

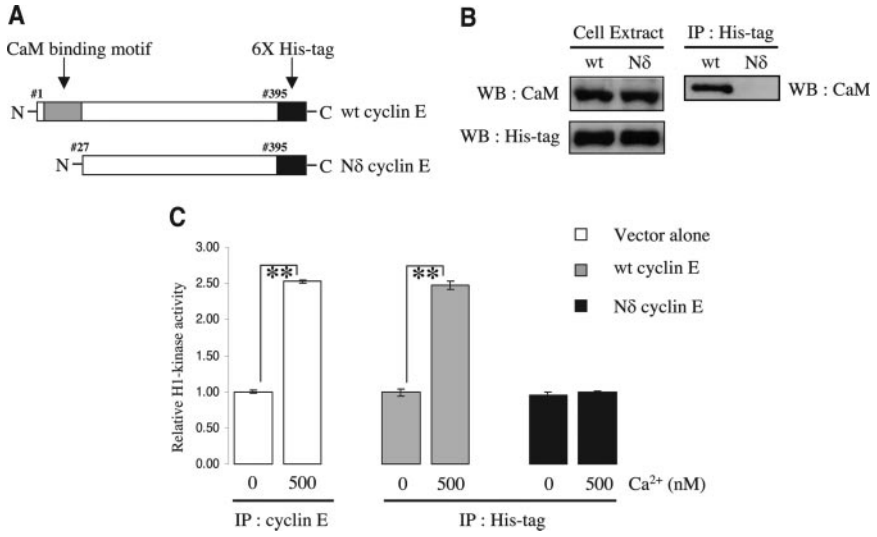

Figure 6. Functional significance of the CaM-binding motif on human cyclin E. A, Diagrammatic representation of His-tagged full-length wt (amino acids 1 to 395) and Nδ mutant (amino acids 27 to 395) forms of human cyclin E are shown. B, Western blot analysis of whole cell extracts for transfected cyclins E (His-tagged wt and Nδ) and CaM and of His-tag–IPd extracts for CaM in G_1/S-synchronized MOVAS. C, H1 kinase assays in G_1/S-synchronized MOVAS stably transfected with vector alone, wt cyclin E, and Nδ cyclin E at 0 and 500 nmol/L [Ca^{2+}]. $^{**}P<0.01$.

calcineurin immunodepletion. Rather, we have identified a physiologically relevant role for CaM at a later and arguably more critical cell cycle checkpoint in VSMC and are the first to directly demonstrate Ca^{2+} sensitivity of its target. Indeed, it is interesting to note that although the studies of Kahl and Means and Taules and colleagues focused on the role of CaM in regulating the early-to-mid G_1 factors cyclin D/CDK4, we have examined the late G_1- and S-phase factors cyclin E/CDK2. Collectively, the key emerging concept from these studies is that CaM mediates Ca^{2+} sensitivities of both the early and late G_1- to S-phase checkpoints through its interactions with cyclin D/CDK4/$p21^{cip1}$ and cyclin E/CDK2/$p27^{kip1}$, respectively.

Although CaM-binding to cyclin E clearly increased the levels of Thr160-phosphorylated (ie, active) CDK2 in extracts from G_1/S-synchronized VSMC (Figure 4B), we have yet to determine how precisely this occurs. Possible explanations include Ca^{2+}/CaM-cyclin E- or Ca^{2+}/CaM-dependent augmentation of (1) another kinase involved in CDK2 activation (namely CDK-activating kinase [CAK], reviewed by Morgan[39]) or (2) the release and/or degradation of the CDK2 inhibitor $p27^{kip1}$ from the otherwise mute cyclin E/CDK2/$p27^{kip1}$ complex. As preliminary data from our laboratory indicate that the binding of CaM to cyclin E does not cause the release of $p27^{kip1}$ from cyclin E/CDK2 complex (data not shown), we suspect that the binding of CaM to cyclin E changes the conformation of the cyclin E/CDK2 complex, such that CDK2 can be more easily phosphorylated by CAK. Activation mechanisms of CaMBPs by CaM strongly support the concept that CaM activates its target proteins by altering their conformation.[40] Further studies will be required to define precisely the mechanisms underlying this observation and the potential involvement of CAK in the Ca^{2+}/CaM-cyclin E-dependent activation of CDK2.

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