Catalytic Activity Is Required for Calcium/Calmodulin-dependent Protein Kinase IV to Enter the Nucleus*

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Calcium/calmodulin-dependent protein kinase IV (CaMKIV) is a nuclear protein kinase that responds to acute rises in intracellular calcium by phosphorylating and activating proteins involved in transcription. Consistent with these roles, CaMKIV is found predominantly in the nucleus of cells in which it is expressed. Here we evaluate nuclear entry of CaMKIV and demonstrate that the protein kinase homology domain is both necessary and sufficient for nuclear localization. Unexpectedly, although catalytic activity is required for nuclear translocation, it is not required for CaMKIV to interact with the nuclear adaptor protein, importin-α. Because the catalytically inactive molecules remain in the cytoplasm, these data suggest that this interaction is not sufficient for nuclear entry. We evaluated a role for other proteins known to interact with CaMKIV in regulation of its nuclear entry. Although our data do not support a role for calmodulin or protein phosphatase 2A, the catalytically inactive CaMKIV proteins interact more avidly with CaM-dependent protein kinase kinase (CaMKK), which is restricted to the cytoplasm. We find that the catalytically inactive proteins do not inhibit nuclear entry of wild-type CaMKIV but do inhibit the ability of the wild-type protein kinase to stimulate cyclic AMP response element-binding protein-mediated transcription. Because activation loop phosphorylation is required for the transcriptional roles of CaMKIV, these data suggest that CaMKK phosphorylation of CaMKIV may occur in the cytoplasm. We propose that sequestration of CaMKK may be the molecular mechanism by which catalytically inactive mutants of CaMKIV exert their “dominant-negative” functions within the cell.

Calcium/calmodulin-dependent protein kinase IV (CaMKIV) is a multifunctional, serine/threonine protein kinase best known for regulation of events that occur in the nucleus (1–3). The biochemistry of CaMKIV has been well studied: upon a transient rise in intracellular calcium, calcium-bound calmodulin (Ca2+/CaM) binds to its autoregulatory domain, which relieves interasteric inhibition and allows intramolecular phosphorylation of multiple serine residues (4–6). This enzyme exhibits low protein kinase activity in vitro (4, 7). An activating protein kinase, calcium/calmodulin-dependent protein kinase kinase (CaMKK), binds to the Ca2+/CaM-CaMKIV complex and phosphorylates CaMKIV on Thr196 in the activation loop (8–10). Activation loop phosphorylation is required to activate CaMKIV fully and render the enzyme capable of phosphorylating nuclear proteins involved in transcription such as the cAMP response element-binding protein (CREB) and the CREB-binding protein (2, 11, 12). After full activation by the three-step mechanism mentioned above, the activity of CaMKIV becomes autonomous and no longer requires bound Ca2+/CaM (13). CaMKIV enzyme activity appears to be negatively regulated by the heterotrimeric protein phosphatase 2A (PP2A), which physically associates with the kinase (14).

The nuclear localization of CaMKIV is well established (3, 14). Immunohistochemistry of endogenous CaMKIV in brain slices illustrates that CaMKIV is concentrated in the nucleus of neurons (15). Immunocytochemistry of cultured cells ectopically expressing CaMKIV also shows nuclear staining (16). However, the mechanism by which CaMKIV enters the nucleus remains to be elucidated. Because CaMKIV is 60 kDa (17), it is unlikely to enter the nucleus by diffusion through the nuclear pore as is typical of proteins less than 40 kDa (18). Rather, it seemed likely that CaMKIV would gain access to the nuclear compartment via the nuclear import machinery and thus would require association with the importin or karyopherin proteins.

We have developed a human embryonic kidney 293A (HEK293A) cell system to evaluate the contributions of the domain structure, catalytic activity, and the known binding partners of CaMKIV to nuclear localization. We find that the C-terminal domain of CaMKIV that contains the CaM binding site and the only candidate nuclear localization sequence (NLS) is not important for nuclear entry. Instead, we find that the kinase homology domain can bind to importin-α and is necessary for nuclear entry. However, importin-α binding is not sufficient for nuclear entry of CaMKIV as full-length proteins with point mutations resulting in kinase inactivation are retained in the cytoplasm. Although phosphorylation of the activation loop is not required for the movement of CaMKIV into the nucleus, LMB, leptomycin B; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEM, minimal essential medium; NLS, nuclear localization sequence; PP2A, protein phosphatase 2A; STAT, signal transducers and activators of transcription; WT, wild-type; YFP, yellow fluorescent protein; CRM1, chromosomal region maintenance export protein 1.
the nucleus, the catalytically inactive CaMKIV proteins bind more avidly to the cytoplasmic CaMkK. Intriguingly, the catalytically inactive mutant kinases inhibit the ability of the wild-type enzyme to drive CREB-mediated transcription in a dose-dependent manner. However, they do not influence the movement of CaMKIV into the nucleus, suggesting that they function in another capacity in the cytoplasm. These results suggest a novel role for protein kinase activity in facilitating the nuclear entry of CaMKIV and provide a mechanistic explanation for the dominant-negative action of the catalytically inactive CaMKIV mutant proteins.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—A HEK293A cell line, obtained from QBI (Quantum Biotechnologies), was used for all cell culture experiments and cultured as described by the company.

Construction of Plasmids—The rCaMKIV cDNA was made using the Stratagene reverse transcription-PCR kit. The RNA was isolated from an adult female Sprague-Dawley rat cerebellum. The entire rCaMKIV construct was verified by sequencing (Amersham Biosciences Thermo Sequenase kit). The rCaMKIV cDNA was subcloned into the pRC-CMV vector. pEYFP-C1-rCaMKIV WT, pEYFP-C1–rCaMKIV 1–314, and pEYFP-C1–rCaMKIV T196A were made by directly subcloning the rCaMKIV cDNA from the pRC-CMV-rCaMKIV WT, rCaMKIV 1–314, and rCaMKIV T196A vectors into the pEYFP-C1 vector (Clontech C-Terminal Enhanced Fluorescent Protein Vector) using BamHI and XbaI sites. The multichaining of pEYFP-C1–pEF-P1–pEF-C1–pEGFP-C1–rCaMKIV WT vectors containing the BstEII and Smal sites within the rCaMKIV cDNA. pEYFP-C1–rCaMKIV 1–36 was made by digesting pEYFP-C1–rCaMKIV WT with BamHI and XbaI, filling in the ends of the remaining plasmid with Klenow, and religating the fragment to create pEYFP-C1–rCaMKIV 1–36. The deletion was confirmed by sequencing; an Arg residue was added following Pro26. pEYFP-C1–rCaMKIV 36–474, pEYFP-C1–rCaMKIV 28–314, and pEYFP-C1–rCaMKIV E207K were made using the Stratagene Site-directed Mutagenesis kit. The introduced deletions/mutations were confirmed by sequencing. SRaCaMKIV was kindly provided by Howard Schulman at Stanford University, pEGFP-HDAC4 and pBJ-HDAC8 were kindly provided by Tso-Pang Yao at Duke University. Glutathione S-transferase (GST) and GST-importin-α expression plasmids were kindly provided by Sally Kornbluth at Duke University. pEBG AMPK kinase-inactive expression plasmid was kindly provided by Lee Witters at Dartmouth University. pCMV-C-Akt hemagglutinin-tagged kinase inactive expression plasmid was kindly provided by the Tsichlis laboratory at the Fox Chase Cancer Center, Philadelphia. pEGFP-β-galactosidase (pHM 829) (19) expression plasmid was kindly provided by Bryan Cullen at Duke University. The pEGFP-NLS expression plasmid was kindly provided by Thomas Brock at the University of Michigan. The FLAG-hCaMKIV WT, FLAG-hCaMKIV K75E, FLAG-hCaMKIV E211K, and FLAG-hCaMKIV T200A expression plasmids were expressed in our laboratory.

Transient Transfections—Transient transfections were performed using LipofectAMINE (Invitrogen) or LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions.

Subcellular Localization Experiments—HEK293A cells were plated at a density of 0.1 × 10^6/35-mm glass-bottomed microwell dish (Mat Tek). The DNA/Opti-MEM ratio was 0.5 μg/l of the indicated constructs (see figure legends) into 50 μl of Opti-MEM (Invitrogen), and the LipofectAMINE/Opti-MEM ratio was 7.5 μl in 50 μl for each 35-mm dish. 36–40 h post-transfection, the cells were viewed by confocal microscopy.

Leptomycin B (LMB) Treatment—Cells were transfected as indicated under “Subcellular Localization Experiments” except that the cells were treated with 10 ng of LMB (Sigma) or an equal volume of MeOH for 3 h before the cells were viewed by confocal microscopy.

Confocal Microscopy—HEK293A cells were plated as described under “Subcellular Localization Experiments.” The living HEK293A cells were incubated with 25 μg/ml DAPI (Sigma) for 1 h. After this incubation, the DAPI was rinsed from the cells, 3 × 1 ml with phosphate-buffered saline, and the cells were viewed using a Zeiss Axiovert 100 confocal microscope and captured using Carl Zeiss Microscope Systems LSM version 3.99 software. The DAPI signal registered on the A_450 channel, and the YFP/GFP proteins were visualized on the A_546 channel. The merged images are false-colored: the DAPI staining in red and the YFP/GFP staining in green.

Transient Transfection, CREB-Luciferase Reporter Gene Assay—HEK293A cells were plated at a density of 0.2 × 10^6/75-mm dish. The DNA/Opti-MEM ratio was 0.4 μg of the indicated protein kinase DNAs (see figure legends) and the additional CREB-luciferase reporter gene DNA mix (0.02 μg of β-galactosidase, 0.16 μg of Gal4-CREB, 0.4 μg of 5× Gal4-TATA-luciferase) into 100 μl of Opti-MEM. The LipofectAMINE/Opti-MEM ratio was 15 μl into 100 μl. The cells were stimulated with 2.5 μM ionomycin (CalBiochem) or an equal volume of dimethyl sulfoxide carrier (Sigma) for 16–20 h and then harvested. Transfections were performed in triplicate for each condition tested on at least three occasions.

Luciferase and β-galactosidase assays were performed as described by Kane and Means (20).

Two-Color Transfections of FLAG-tagged CaMKIV for Protein Production from HEK293A Cells—HEK293A cells were plated at a density of 1.6 × 10^5/100-mm dish. The DNA/Opti-MEM ratio was 4.0 μg of the indicated DNAs (see figure legends) into 400 μl. The LipofectAMINE 2000/Opti-MEM ratio was 15 μl into 400 μl. The cells were harvested 16 h post-transfection.

Purification of FLAG-tagged CaMKIV from Transiently Transfected HEK293A Cells—The cells were harvested by rinsing them once with Hank’s balanced saline solution without metals (Invitrogen) then scraped into 1 ml of Nonidet P-40-based lysis buffer (2 mM EGTA, 2 mM EDTA, 25 mM Tris, pH 7.5, 0.5% Nonidet P-40, 50 mM NaCl, 25 mM NaH2PO4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 100 mM sodium orthovanadate, 1 mM okadaic acid, 10 mM EDTA, 25 mM NaCl, 25 mM Tris, pH 7.4). The FLAG peptide (Sigma) was added to the TBS-washed resin to a final concentration of 300 ng/ml in a reaction volume of 50 μl. The resin was incubated on ice with occasional flicking for 1 h. The eluted FLAG-tagged CaMKIV was recovered in the supernatant by spinning the sample at 1,000 × g for 5 min.

Immunoprecipase Gel Analysis—Cell lysates were harvested in the Nonidet P-40-based lysis buffer as described above. Either 5 μl of crude cell lysate, or 20 μl of purified lysate was resolved by 10% PAGE. The resolved proteins were stained with Coomassie Blue (21).

Immunoblot Analysis—Cell lysates were harvested and electrophoresed as described above. The resolved proteins were transferred from the gels to an Immobilon-P membrane (Millipore). There they were probed with the following antibodies: α-CaMKIV (BD Biosciences), α-P2PA (BD Biosciences), α-CaMKK (BD Biosciences), α-GFP (Santa Cruz Biotechnologies, Inc.), α-FLAG-m2 (Sigma), and α-CaMKI (generated by C. R. Kahl in Dr. Anthony Means’s laboratory at Duke University) at a 1:1,000 dilution. The following antibodies used secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.): α-CaMKIV, α-P2PA, α-CaMKK, and α-FLAG-m2. α-GFP and α-CaMKI used α-rabbit secondary antibodies at a 1:5,000 dilution (Jackson ImmunoResearch Laboratories, Inc.). All immunoreactivity was detected using ECL (Amersham Biosciences).

γ-1CaM overlay assay was performed as described by Anderson et al. (9).

In Vitro Binding Assay with GST Fusion Proteins—Wild-type and mutant recombinant FLAG-tagged CaMKIV proteins were expressed in HEK293A cells as described above. GST or GST-importin-α was produced and purified using glutathione-Sepharose resin as described by Moore et al. (22). 200 ng of each purified GST chimeric protein was mixed with the purified FLAG-tagged CaMKIV proteins in 500 μl of 50 mM Tris, pH 7.0, 40 mM NaCl, 0.5% Nonidet P-40, and the proteoliposome assay was performed at 4 °C for 2 h with rotation. The beads were then washed four times with 1 ml of Nonidet P-40-based lysis buffer and suspended in 50 μl of SDS-PAGE sample buffer (21). The bound proteins were detected by immunoblotting, α-FLAG-m2 antibody.

In Vitro Phosphorylation Assays—The ability of purified CaMKIV and CaMKKβ to phosphorylate purified CReB, importin-α, or importin-β in vitro was assayed as follows. 75 ng CaMKIV, 200 ng of CaMKKβ, or 75 ng of CaMKIV and 200 ng of CaMKKβ were preincubated in kinase buffer (5 mM Tris, pH 7.5, 200 μM ATP, 1 mM dithiothreitol, 10 mM MgCl2, 1 mM CaCl2, 0.1% Tween 20, and 1 μM CaM) in a volume of 30 μl. Preincubation consisted of 200 μM cold ATP and the proteins for 20 min with rotation. Purified fully active 200 μM CaMK IV with CaMKK without labeling it. Then, the incubated reactions were initiated by the addition of 0.2 μl of [γ-32P]ATP (10 μCi/mmol) and 2.5 μg of the indicated substrate: CReB, importin-α, or importin-β. After the 10-min reaction at 30 °C, the reactions were terminated with the addi-
Coomassie Blue staining. The incorporation of 32P into the protein was resolved using 10% SDS-polyacrylamide gels and were visualized by autoradiography. For Western blotting, the proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with CaMKIV antibodies. The membrane was then incubated with 32P-labeled ATP to detect the incorporation of phosphate into the protein. The proteins were visualized by autoradiography. The incorporation of 32P into the protein substrates was determined by autoradiography.

RESULTS

Development of a Cell-based System to Investigate the Subcellular Localization of CaMKIV—Although CaMKIV is predominantly localized to the nucleus, the mechanism of its localization remains to be elucidated. To evaluate this process, we examined the subcellular localization of ectopically expressed CaMKIV in HEK293A cells. Because it is unknown how mutations made within the CaMKIV proteins affect its interaction with the CaMKIV antibodies, we added an YFP tag at the N terminus of CaMKIV. As shown in Fig. 1A, overexpressed YFP-tagged wild-type CaMKIV localizes to nuclei of living HEK293A cells. We expressed the YFP tag alone or fused either to the NLS of the SV40 large T antigen or to β-galactosidase to determine whether the YFP tag influenced subcellular localization of fusion proteins. Expression of these fusion proteins demonstrated that YFP alone was distributed uniformly throughout the cell (Fig. 1B). Adding the SV40 large T antigen NLS resulted in accumulation of the GFP fusion protein in the nucleus (Fig. 1C), and the GFP-β-galactosidase fusion protein remained in the cytoplasm (Fig. 1D). Based on these observations, we concluded that YFP-CaMKIV fusion proteins would accurately reflect the intracellular localization of CaMKIV and CaMKIV mutant proteins.

The Catalytic Domain of CaMKIV Is Necessary and Sufficient to Direct the Nuclear Localization of CaMKIV—Because CaMKIV is a ~60-kDa protein (17), it is not predicted to diffuse freely into the nucleus. Proteins greater than 40 kDa containing a polybasic NLS, like the prototypical NLS (PKKKRKV) of the SV40 large T antigen, bind to importin-α and are actively transported into the nucleus (23–25). We examined the amino acid sequence of CaMKIV for the presence of a canonical NLS and found that the only candidate NLS was a portion of the CaM binding domain (ARRKLK323). Therefore, we generated truncation mutants of the wild-type protein, each fused to YFP, and visualized their subcellular localization by confocal microscopy. Fig. 2A is a schematic representation of the regions each truncation protein encompassed and indicates the fragment containing the putative NLS.

The first 314 amino acids of CaMKIV contain both the serine-rich N terminus and the protein kinase domain. As shown in Fig. 2B, this protein fragment entered the nucleus as a YFP fusion protein. We created two additional YFP fusion proteins to separate the serine-rich N terminus from the kinase homology domain to determine which was required for nuclear localization of CaMKIV. Our observations showed that YFP fused to the serine-rich N terminus distributed uniformly throughout the cell (Fig. 2B). In contrast, YFP fused to the kinase homology domain, CaMKIV 28–314, generated a nuclear-targeted protein kinase (Fig. 2B). These results suggested that the kinase homology domain was sufficient for nuclear entry of CaMKIV.

We generated a YFP-tagged version of the C-terminal 168 amino acids (CaMKIV 306–474) and examined its localization in cells to be certain that the catalytic domain was not only sufficient but also necessary for nuclear entry. As seen in Fig. 2B, this fusion protein localized to the cytoplasm. These results were contrary to what we predicted because the putative NLS resides in the C-terminal 168 amino acids (ARRKLK323) and did not function as a NLS in the context of the truncated molecule. Thus, the protein kinase domain is both necessary and sufficient to direct nuclear entry of CaMKIV as a YFP fusion protein.

Catalytic Activity Is Required for CaMKIV to Enter the Nucleus—Because of our findings that the protein kinase domain targeted CaMKIV to the nucleus, we questioned whether the activity of the protein kinase domain was required for nuclear translocation of CaMKIV. Catalytic activity of CaMKIV can be prevented by introduction of a single point mutation into the ATP binding domain. Therefore, we introduced this mutation (K71M) into the N-terminal 1–314 fragment and assessed its subcellular localization by confocal microscopy. This construct did not express well and proved deleterious for cellular survival. Therefore, we conducted all of our subsequent studies using mutations introduced into the full-length enzyme.

We mutated the ATP binding domain (K71M) in the context of the full-length enzyme and evaluated its expression by immunoblotting. As seen in Fig. 3A, YFP-CaMKIV K71M was inactive in a CREB-mediated transcription assay. Therefore, we examined its subcellular localization by confocal microscopy. Viewing the cells revealed that this catalytically inactive mutant protein kinase (K71M) was confined to the cytoplasm (Fig. 3A) which supported the idea that catalytic activity was required for nuclear localization.

To confirm the notion that catalytic activity was required for nuclear entry, we mutated the catalytic glutamate residue (Glu207) in the large lobe of the protein kinase. Based on structural analysis of other protein kinases, Glu207 was predicted to be responsible for stabilizing the internal charge of the catalytic lysine (Lys71) when the γ-phosphate of ATP is transferred to the protein substrate (26, 27). The YFP-CaMKIV E207K fusion protein was inactive in a CREB-mediated transcription assay (Fig. 3B). This catalytically inactive mutant protein kinase (E207K) also localized to the cytoplasm (Fig. 3A). These results confirm that nuclear entry of full-length CaMKIV required not only the presence of the protein kinase domain but...
also its intrinsic catalytic activity. Because kinase-competent CaMKIV molecules are substrates for the CaMKKs, we investigated whether phosphorylation of the activation loop (Thr<sup>196</sup>) was required for nuclear entry. Strikingly, the activation loop mutant, YFP-CaMKIV T196A, localized to the nucleus (Fig. 3A) but did not drive CREB-mediated transcription (Fig. 3B), confirming previous results (9). These observations demonstrated that although catalytic activity was required for nuclear entry, phosphorylation of the activation loop was not.

LMB Failed to Alter the Subcellular Localization of Catalytically Inactive CaMKIV Mutant Protein Kinases—Because catalytically inactive YFP-CaMKIV proteins reside in the cytoplasm, we speculated that catalytic activity might control nuclear export rather than nuclear import. To address this question, cells expressing either wild-type or catalytically inactive YFP-CaMKIV were treated with LMB, a CRM1-mediated nuclear export inhibitor, and the subcellular localization of these proteins was visualized by confocal microscopy. As shown in Fig. 3C, treatment of cells with LMB for 3 h failed to alter the cytoplasmic localization of either the K71M or the E207K mutant CaMKIV protein. Fig. 3D verified the potency of the LMB by demonstrating that GFP-HDAC4, a protein that is exported in a CRM1-dependent manner, was only observed in the nucleus in the presence of the drug (28). This finding was in stark contrast to the behavior of the wild-type YFP-CaMKIV protein kinase, which localized entirely to the nucleus even in the absence of LMB (Fig. 3C). Therefore, if the inactive CaMKIV mutant proteins do shuttle between nucleus and cytoplasm, they must do so very rapidly and in a CRM1-independent manner.

Interaction of CaMKIV, but Not CaMKI, with Importin-α—Nuclear proteins that are too large to diffuse passively through the nuclear pore are transported actively into the nucleus by a family of proteins known as the importins or karyopherins (29). After transfection with wild-type CaMKIV, we prepared HEK293A cell lysates and incubated them with GST or GST-importin-α beads to assess whether CaMKIV bound importin-α. As seen in Fig. 4A, overexpressed untagged CaMKIV associates with GST-importin-α.

Because the primary structure of the catalytic domain of CaMKIV is 67% similar to a closely related cytoplasmic family member, CaMKI, and catalytically inactive CaMKIV molecules remain in the cytoplasm, we evaluated whether the binding of CaMKIV to the importins was a general property of the CaM kinases. Using a GST pull-down assay, we investigated whether ectopically expressed CaMKI bound to importin-α. As seen in Fig. 4A, CaMKI was unable to associate with GST-
importin-α, suggesting that CaMKIV must interact with the importins through a region of the protein kinase that is specific to CaMKIV.

Next, we transiently transfected HEK293A cells with the YFP-CaMKIV deletion mutations described in Fig. 2A to determine the region of CaMKIV required for binding to importin-α. As seen in Fig. 4B, the catalytic domain that localized to the nucleus also bound to GST-importin-α. Furthermore, we overexpressed either wild-type or catalytically inactive CaMKIV FLAG-tagged proteins in HEK293A cells, purified them, and then incubated the proteins with GST or GST-importin-α beads. As seen in Fig. 4C, the wild-type and catalytically inactive protein kinases bound similarly to importin-α. FLAG-tagged HDAC6 was included in this assay to demonstrate that the interaction of CaMKIV with the importins was not caused by the presence of the FLAG tag at the N terminus of these fusion proteins. These results revealed that although CaMKIV bound the importins via its kinase domain, catalytic activity was not required for this interaction. Thus, we conclude that although it may be necessary, importin binding is not sufficient for the nuclear entry of CaMKIV.

CaMKIV Does Not Phosphorylate the Importins—Because only catalytically competent CaMKIV molecules are able to enter the nucleus, we tested whether phosphorylation of either importin-α or importin-β was necessary for the nuclear entry of CaMKIV. To address this problem, CaMKIV, CaMKKβ, CREB, and the importins were made in bacteria. The proteins were purified and then mixed together in a kinase assay under standard conditions. As shown in Fig. 4D (upper panels), recombinant CaMKIV that has been activated by CaMKKβ was able to phosphorylate CREB, a known protein substrate. However, Fig. 4D also demonstrates that neither importin-α nor importin-β is phosphorylated by CaMKIV, eliminating the possibility that CaMKIV must phosphorylate the importins for it to gain access to the nucleus. Fig. 4D (lower panels) is included to demonstrate the abundance of substrate molecules present in each kinase assay.

The Physical Association among CaMKIV, CaM, PP2A, and CaMKK Is Insufficient for Wild-type CaMKIV to Enter the Nucleus—The first step in the enzymatic activation of CaMKIV is its binding to Ca²⁺/CaM. To determine whether Ca²⁺/CaM binding to CaMKIV was required for CaMKIV to enter the nucleus, we analyzed the ability of the YFP-tagged wild-type and catalytically inactive CaMKIV protein kinases to bind 125I-CaM in an overlay assay. As shown in Fig. 5A, Ca²⁺/CaM binds equivalently to both the wild-type and the catalytically inactive CaMKIV protein kinase molecules, suggesting that Ca²⁺/CaM binding alone is insufficient to confer nuclear localization of CaMKIV.

Because PP2A binds CaMKIV and has been proposed to

**Fig. 3.** Subcellular localization of the CaMKIV activity mutant proteins and their insensitivity to LMB. A, HEK293A cells transiently transfected with DNA encoding YFP-tagged CaMKIV point mutations were visualized by confocal microscopy. B, HEK293A cells transiently transfected with the CREB-luciferase reporter gene DNA mix and DNA encoding YFP-tagged versions of wild-type (WT) or mutated CaMKIV were treated with tiamonycin (gray bars) or dimethyl sulfoxide (white bars); the luciferase activity is reported as relative luciferase light units.

**Fig. 4.** GST pull-down and kinase assay of CaMKIV and importin-α. The interactions between endogenous CaMKIV, ectopically expressed CaMKI, or the YFP-tagged CaMKIV deletion mutants described in Fig. 1 and importin-α were assessed using HEK293A cell lysate and GST or GST-importin-α beads. The interactions were evaluated by immunoblot using antibodies specific to CaMKIV (A, left), CaMKI (A, right), or YFP (B). The interactions between FLAG-tagged purified wild-type or mutant CaMKIV or FLAG-tagged HDAC6 proteins and importin-α were assessed using HEK293A cell lysates and GST-importin-α beads. The interactions were evaluated by immunoblot using antibodies specific to the FLAG epitope (C). Phosphorylation of CREB S142A (left), importin-α (center), or importin-β (right), by CaMKIV, CaMKKβ, or both CaMKIV and CaMKKβ was determined by autoradiography (D, top panel). The corresponding Coomassie Blue-stained membranes are shown below (D, lower panel).
regulate its activity negatively, we investigated the role of this protein-protein interaction on the subcellular localization of CaMKIV (30). Immunoblot analysis demonstrated that immunoprecipitated ectopically expressed FLAG-tagged wild-type and catalytically inactive CaMKIV protein kinases both interacted with endogenous PP2A (Fig. 5C). This observation demonstrates that PP2A binding is not sufficient for CaMKIV to enter the nucleus.

Tokumitsu et al. (10) used a GST pull-down assay to show that recombinant GST-CaMKIV interacted with overexpressed CaMKK in COS-7 cell lysates. Therefore, we assessed the relevance of this protein-protein interaction to the subcellular localization of CaMKIV. Immunoblot analysis demonstrated that both FLAG-tagged wild-type and catalytically inactive CaMKIV interacted with ectopically expressed CaMKKKβ in HEK293A cell lysate (Fig. 5B). The FLAG-T200A mutant protein kinase interacted weakly with CaMKKKβ, which was in stark contrast to the robust interaction seen between either the FLAG-CaMKIV K75E or the FLAG-CaMKIV E211K mutant protein kinase and CaMKKKβ. We conclude from these results that CaMKKKβ binding to CaMKIV was not sufficient for CaMKIV to enter the nucleus. Indeed, CaMKKKβ and the catalytically inactive protein kinases are cytoplasmic enzymes, and our data suggest that in the absence of a functional kinase domain, CaMKIV may remain associated with CaMKKKβ in the cytoplasm. Fig. 5D is included to demonstrate that the level of protein expression of each CaMKIV mutant protein kinase was similar.

**The Catalytically Inactive Mutant, K71M, Inhibits the Ability of the Wild-type Enzyme to Drive CREB-mediated Transcription, but It Does Not Inhibit Its Nuclear Localization**—Although the catalytically inactive mutant protein kinases do not alter the subcellular localization of wild-type CaMKIV, they could exhibit dominant-negative activity by competing with the wild-type enzyme to stimulate CREB-mediated transcription. As seen in Fig. 6A, either the CaMKIV K71M or the CaMKIV E207K catalytically inactive mutant proteins reduced the ability of the wild-type enzyme to drive transcription. We next evaluated this inhibitory effect as a function of K71M concentration. Fig. 6B shows a dose-dependent effect of K71M to inhibit transcription by a fixed concentration of WT CaMKIV. Similar results were observed with the E207A mutant (data not shown). Taken together our data suggest that the catalytically inactive CaMKIV proteins exert their dominant-negative effects by sequestering a cytoplasmic component(s) required to facilitate the ability of CaMKIV to stimulate CREB-mediated transcription. Based on the results presented in Fig. 5B, we speculate that CaMKKK is at least one such sequestering component.

To investigate whether the underlying mechanism by which the catalytically inactive CaMKIV protein kinase molecules functioned was by competing for cytosolic factors that aided in the nuclear entry of kinase-competent CaMKIV, we overexpressed a fixed amount of YFP-tagged wild-type CaMKIV and
increasing amounts of FLAG-tagged catalytically inactive CaMKIV. As shown in Fig. 6C, increasing the amount of FLAG-tagged CaMKIV K75E DNA does not alter the nuclear localization of the wild-type protein. Immunoblot analysis of the cell lysates demonstrated that varying the amounts of YFP-tagged wild-type CaMKIV and FLAG-tagged CaMKIV K75E DNA added to the cells produced a corresponding amount of protein (Fig. 6D). Therefore, we eliminated the possibility that the mechanism by which catalytically inactive CaMKIV functioned as a dominant-negative molecule was by inhibiting the nuclear localization of the wild-type enzyme.

To evaluate the possibility that the apparent increased interaction between CaMKKK and the catalytically inactive CaMKIV mutant proteins provided the mechanism that precluded the entry of the catalytically inactive mutant proteins into the nucleus, we conducted a competition experiment. In this experiment we overexpressed a fixed amount of YFP-tagged rCaMKIV K71M (the equivalent rat mutant to human K75E) and increasing amounts of FLAG-tagged wild-type CaMKIV. As shown in Fig. 6E, increasing the amount of FLAG-tagged wild-type CaMKIV DNA by a factor of 5 (from 0.18 to 0.71 μg) does not alter the cytoplasmic localization of the YFP-tagged CaMKIV K71M. Immunoblot analysis of cell extracts reveals that the amount of wild-type protein present in the cells was indeed several times the amount of the mutant protein (data not shown). We conclude that the inability of the catalytically inactive CaMKIV mutant proteins to enter the nucleus is unlikely to be caused by the increased interaction with CaMKKK.

**DISCUSSION**

The present study shows that CaMKIV protein kinase activity is required for nuclear entry. This was unanticipated because a catalytically inactive mutation (K42M) in the closely related CaMKII protein kinase does not affect its nuclear localization.3 Mutations within either the large lobe (E207K) or the small lobe (K71M) of the kinase domain inhibit the catalytic activity of CaMKIV and cause CaMKIV to remain in the cytoplasm (Fig. 3B). These results are consistent with our subcellular localization analysis of the various truncated forms of CaMKIV. Through the creation of YFP-CaMKIV fusion proteins, we have demonstrated that the catalytic domain is sufficient for nuclear entry. The cytoplasmic localization of the C-terminal domain confirms this observation and provides further evidence that the N-terminal half of CaMKIV is both necessary and sufficient for nuclear entry of CaMKIV. These observations demonstrate that catalytic activity is required for nuclear localization of CaMKIV and raise several possible explanations that we have considered and attempted to address experimentally.

First, we considered the possibility that catalytic activity is required for autophosphorylation of the kinase which in turn facilitates nuclear entry. There are several published examples where the phosphorylation of a protein regulates its subcellular localization. Phosphorylation of the yeast transcription factor Pho4 is required for Pho4 to bind to Msn5, a yeast export receptor (31, 32). The STAT family of transcription factors does not exist for CaMKIV.

An important issue related to the mechanism of CaMKIV nuclear entry is the spatial discrepancy between this nuclear kinase and its activating kinase, CaMKK, which has only been detected in the cytoplasm (3, 38–40). Biochemical data demonstrate that CaMKIV is a substrate for CaMKKβ in vitro and that CaMKKβ enhances the ability of CaMKIV to drive CREB-mediated translocation in vivo (9). Taken together, these results present a conundrum because the activating kinase seems to be spatially separated from its substrate by the nuclear membrane. Several possibilities can be considered to resolve this conundrum. First, CaMKKK could transiently enter the nucleus and phosphorylate CaMKIV when the latter pro-

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3 H. Schulman, personal communication.
tein is restricted to this compartment or CaMKIV could cycle out of the nucleus, become phosphorylated in the cytoplasm by CaM KK, and return to the nucleus. However, our data have shown that neither CaMKIV nor CaM KK is sensitive to LMB,4 the only known inhibitor of nuclear export. Therefore, to test this model we will need either to identify the nuclear transport machinery each uses to cycle, or create mutations within the kinases that reduce their rates of cycling. Second, CaMK IV could phosphorylate CaMK IV in the cytoplasm before it enters the nucleus. Although this may occur, the observation that the activation loop mutant (T196A) enters the nucleus similar to the wild-type enzyme shows that the phosphorylation event is not a requirement for nuclear entry. If activation loop phosphorylation does occur in the cytoplasm before nuclear entry, its nuclear localization might dictate substrate preference. On the other hand, if phosphorylation of CaMK IV occurs in the cytoplasm, then activation of the kinase would be dependent on a nuclear Ca2+ signal as suggested by the experiments of Chawla et al. (1998) (42). Lastly, cytoplasmic CaM KK could activate Factor X, thereby enabling it to enter the nucleus and phospho-

...cellular localization, the catalytically inactive CaMK IV domain has been shown to be both necessary and sufficient for activity than does the wild-type kinase (Fig. 5). This indicates that AKT kd and AMPK-kd do not compete with wild-type CaM KK for CaM KK accessibility in the way that catalytically inactive CaM KK mutants do. This is consistent with work by Tokumitsu (10) demonstrating that mutations of CaM KK which are unable to bind and activate CaM KK are still able to activate AKT. In this way, the dominant-negative action of catalytically inactive CaM KK exhibits specificity for the CaM cascade.

In conclusion, our results show that CaM KK protein kinase activity is required to enter the nucleus, although no functional NLS has been identified in CaM KK. Indeed, the catalytic domain has been shown to be both necessary and sufficient for nuclear translocation. Although the interaction between CaM KK and CaM or PP2A does not seem to influence its subcellular localization, the catalytically inactive CaM KK protein kinase does show enhanced binding to CaM KK. This increased avidity for interaction is not likely to be mechanism by which the catalytically inactive molecules are excluded from the nucleus, but it may explain how the catalytically inactive molecules function as dominant-negative proteins within the cell. These studies raise new questions for future experimentation namely, 1) is the catalytic activity of CaM KK required for its autophosphorylation or its interaction with another protein in the cell, and 2) what, in addition to importin binding, is required for CaM KK to translocate into the nucleus?

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4 E. E. Corcoran, S. M. Lemrow, and A. R. Means, unpublished observation.
Catalytic Activity Is Required for Calcium/Calmodulin-dependent Protein Kinase IV to Enter the Nucleus
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