Phosphorylation at the Nuclear Localization Signal of Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase II Blocks Its Nuclear Targeting

(Received for publication, November 21, 1997, and in revised form, May 6, 1998)

E. Kevin Heist‡‡, Mallika Srinivasan‡, and Howard Schulman¶

From the Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305-5125

Translocation of protein kinases with broad substrate specificities between different subcellular compartments by activation of signaling pathways is an established mechanism to direct the activity of these enzymes toward particular substrates. Recently, we identified two isoforms of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaM kinase II), which are targeted to the nucleus by an alternatively spliced nuclear localization signal (NLS). Here we report that cotransfection with constitutively active mutants of CaM kinase I or CaM kinase IV specifically blocks nuclear targeting of CaM kinase II as a result of phosphorylation of a Ser immediately adjacent to the NLS of CaM kinase II. Both CaM kinase I and CaM kinase IV are able to phosphorylate this Ser residue in vitro, and mutagenesis studies suggest that this phosphorylation is both necessary and sufficient to block nuclear targeting. Furthermore, we provide experimental evidence that introduction of a negatively charged residue at this phosphorylation site reduces binding of the kinase to an NLS receptor in vitro, thus providing a mechanism that may explain the blockade of nuclear targeting that we have observed in situ.

Phosphorylation and dephosphorylation reactions control a myriad of signal transduction processes within the cell including cell growth and differentiation, metabolic pathways, and gene expression. The specificity of some kinases mediating these reactions is attained by a strict substrate specificity that limits the action of these dedicated kinases to a single or limited number of potential targets. Other kinases, however, are able to phosphorylate a large number of proteins in vitro, so the in vivo specificity of these kinases must occur through a different mechanism. Examples of these multifunctional or general protein kinases include protein kinase A, protein kinase C, and the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaM kinase)\textsuperscript{1} family consisting of CaM kinase I, CaM kinase II, and CaM kinase IV (reviewed in Refs. 1 and 2). All of these kinases are able to phosphorylate nuclear transcription factors in vitro at sites that either activate or repress gene expression and so all of these kinases have the capacity, at least theoretically, to alter cellular phenotype through changes in protein expression. Over the past decade, the evidence that this actually occurs in vivo is becoming increasingly strong; for example, there is now abundant evidence that protein kinase A mediates activation of the cyclic AMP response element-binding protein through phosphorylation of a key Ser residue (3, 4).

Nuclear localization of a kinase is necessary for phosphorylation of nuclear proteins such as transcription factors, although there are examples of transcription factors that are activated in the cytoplasm and then translocate to the nucleus (5, 6). The ability of the catalytic subunit of protein kinase A to be released from cytoplasmic tethering and then passively diffuse into the nucleus, where it can phosphorylate nuclear proteins such as cyclic AMP response element-binding protein, has been described (7). More recently, active export of protein kinase A out of the nucleus under certain conditions based on a nuclear export signal has also been reported (8). Regulation of protein kinase C localization appears to occur, at least in part, through isoform-specific targeting to anchoring proteins that are presumably located near its substrates (9). CaM kinase I has been localized predominantly to the cell cytoplasm, while CaM kinase IV is predominantly nuclear (10, 11), although the mechanisms governing the subcellular localization of these two kinases have not yet been elucidated.

Isoform-specific targeting of CaM kinase II to the nucleus based on an alternatively spliced nuclear localization sequence (NLS) has been described (12, 13). CaM kinase II is composed of a multigene family derived from four related genes: \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \) (reviewed in Refs. 1 and 2). The \( \alpha \), \( \beta \), and \( \gamma \) isoforms of CaM kinase II all share a common core NLS amino acid sequence, KKRK, which is homologous to the NLS of the SV40 large T antigen (12). Nuclear localization of the \( \delta \) and \( \alpha \) isoforms of CaM kinase II both in situ and in vivo has been reported (12, 13); \( \gamma \) is also presumably a nuclear isoform, although this has not been investigated. Other isoforms of CaM kinase II do not contain any known NLS sequence and localize to the cytoplasm. Interestingly, CaM kinase II forms dimers of 8–12 subunits in situ and in vivo, and the final localization of the multimer to the nucleus or cytoplasm appears to be based on whether it is composed of predominantly nuclear or cytoplasmic isoforms (12). These dimers are of sufficient size (400–600 kDa) that any transport of intact holoenzyme across the nuclear membrane is likely to require an active, nuclear export signal or NLS-dependent process. Immunostaining of brain sections has demonstrated nuclear localization of CaM kinase II in regions with high expression levels of nuclear forms and cytoplasmic localization of the kinase in regions expressing predominantly cytoplasmic forms (13). Thus, like the other multifunctional protein kinases, CaM kinase II does appear to have access to the cell nucleus, but such access depends on the type and ratio of isoforms expressed.
Many different nuclear events have been ascribed to CaM kinase II. This kinase is capable of regulating nuclear envelope breakdown in sea urchin oocytes (14), releasing Xenopus oocytes from meiotic metaphase arrest (15), promoting the maturation of oocytes (16), and blocking the cell cycle at the G2 to M transition (17). CaM kinase II has also shown to activate the CCAAT enhancer element-binding protein β (CEBPβ) (18) and recently has been shown to block activity of the transcription factor cyclic AMP response element-binding protein and either block or activate the related transcription factor ATF-1 through phosphorylation of both positive and negative regulatory sites (19–21). Many of these studies utilized a truncated mutant of CaM kinase II, which does not associate into holoenzymes and is able to diffuse freely between the nucleus and cytoplasm as a result of its small size. Presumably, nuclear localization of CaM kinase II would be required in order to mediate processes involving phosphorylation of nuclear transcription factors. A recent study demonstrates that, among three transfected isomers of CaM kinase II, only the nuclear-targeted isoform is able to activate an atrial natriuretic factor promoter construct in cultured ventricular myocytes, while the two cytoplasmic isoforms of CaM kinase II do not affect expression of this construct (22).

Recently, autophosphorylation of cytoplasmic CaM kinase II was shown to cause translocation of the kinase to the postsynaptic density in cultured hippocampal neurons (23). We are interested in whether there are mechanisms other than isoform-specific expression that can affect the ability of CaM kinase II to localize to the nucleus and, by doing so, be available to translate Ca2+ elevations into the processes described above. The capability of many NLS-containing proteins to translocate to the nucleus is affected either positively or negatively by phosphorylation (reviewed in Ref. 24). We have studied the ability of the other multifunctional kinases, protein kinase A, protein kinase C, CaM kinase I, and CaM kinase IV, to alter nuclear targeting of CaM kinase II through phosphorylation. We report here that CaM kinase I and CaM kinase IV are capable of phosphorylating a Ser residue immediately adjacent to the NLS of αp- and δp-CaM kinase II and that this phosphorylation causes reduced binding to an NLS receptor and blocks targeting of CaM kinase II to the cell nucleus.

**Experimental Procedures**

**Construction of CaM Kinase II Mutants**—The cloning of the α, αp, and δp isoforms of CaM kinase II into the SGs eukaryotic expression vector has been described previously (12, 13, 25). Mutants of αp-CaM kinase II incorporating the following underlined Ser to Ala and Ser to Thr mutations to the NLS of protein kinase C, CaM kinase I, and CaM kinase IV, to alter ability of the other multifunctional kinases, protein kinase A, to the nucleus is affected either positively or negatively by the underlined mutations. A mutant incorporating the NLS of kinase II, and that this phospho-

**Construction of CaM Kinase I, CaM Kinase IV, and, and Calsmodulin Expression Constructs—**Human CaM kinase I clones, including 1) a full-length, wild-type clone, 2) a Ca2+-/calmodulin-independent, truncated clone coding for residues 1–294, and 3) these same two constructs with an activating T177D mutation were a generous gift from A. Means (Duke University) (28). In order to clone these constructs into the SRL expression vector, the Pee site of SRL was cleaved and blunted with Klenow fragment, and an oligonucleotide linker containing a BglII site (Life Technologies, Inc.) was ligated into this site by standard techniques. The CaM kinase I clones were then cut with BamHI and EcoRI, producing fragments encoding the entire kinase reading frame, which was then cloned into the EcoRI and BglII sites of SRL. Rat CaM kinase IV, including a full-length, wild-type clone as well as a Ca2+-independent truncated clone coding for residues 1–313 cloned into a PcmV expression vector was also a gift of A. Means (29). The full reading frame for CaM kinase IV was cloned out of this vector by cutting at the 5′-end of the coding region with NcoI, blunting this site with T4 DNA polymerase, and then cutting the 3′-end with ApaI. This fragment was then cloned into the EcoRI and ApaI sites of SRL. A clone containing the entire reading frame of human calmodulin was a generous gift of P. Yaswen. SRL-calmodulin was created by cutting the 5′-end of this clone with NcoI, bluntng with Klenow fragment, cutting the 3′-end with KpnI, and then cloning this fragment into the EcoRV and KpnI sites of SRL. All subcloning procedures were performed according to standard techniques, and all enzymes and buffers were obtained from Life Technologies and New England Biolabs Inc. SRL-protein kinase A was a gift of T. Soderling (25). This was confirmed using the methods of Mannheim and Maniatis (26) and used for transfections according to the manufacturer's protocols. Cells were plated onto 35-mm cell culture dishes (Falcon) on the day prior to transfection at a density such that they were approximately 70% confluent on the day of transfection. For each transfection, 1.5–1.75 μg of total DNA was combined with 5 μl of Lipofectamine (Life Technologies, Inc.), and 0.5 mg of CaM kinase II was cotransfected with 0.5 μg of CaM kinase II and继续基本的clone coding for residues 1–313 cloned into a SRL expression vector was also a gift of A. Means (29). Cell Culture and Transfection— COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% supplemented calf serum (HyClone Labs) in a 10% CO2 incubator as described previously (25). Transfections were carried out using the protocols of Gall排除和 Morita-Bоор (25) and transfected cells were harvested for analysis 24 h post-transfection. For immunofluorescence studies, 0.25 μg of total DNA was combined with 5 μl of Lipofectamine (Life Technologies, Inc.), and 0.5 μg of CaM kinase II was cotransfected with 0.5 μg of SRL vector, SRL-CaM kinase I, or SRL-CaM kinase IV. For cotransfections including calmodulin and/or CaM kinase II, 0.25 μg of CaM kinase II was cotransfected with 0.5 μg of CaM kinase I or IV with or without 0.5 μg of SRL-calmodulin with or without 0.5 μg of CaM kinase kinase. For NLS binding studies, 1.5 μg of CaM kinase II was used for transfection.

**Immunocytofluorescence**—Fixation and staining of COS-7 cells were performed on 35-mm tissue culture dishes as described previously (12). Immunodetection of α-CaM kinase II and αp-CaM kinase II was performed with a monoclonal antibody that recognizes both isoforms of CaM kinase II. Immunodetection of hemagglutinin-tagged CaM kinase II was performed with a monoclonal antibody to this tag (Boehringer Mannheim). Secondary antibody detection with a rhodamine-linked goat anti-mouse antibody and fluorescence microscopy and photograpghy were performed as described (12). In order to quantitate nuclear localization of the kinase, 100 transfected cells were scored in a blinded fashion as to whether the kinase was predominantly nuclear or predominantly cytoplasmic. The identity of the nucleus was verified by comparative phase-contrast microscopy. Cells in which definitive localization of CaM kinase II staining could not be established (a small fraction of the total) were omitted from analysis.

**Peptide Phosphorylation**—Phosphorylated CaM kinase I was a generous gift
of A. Nairn (Rockefeller University), and purified CaM kinase IV was a generous gift of A. Means. Peptides with the amino acid sequences GGKKRRSSSSVQME and GGKKRRSSSSVQME were generated on an automated peptide synthesizer. Phosphorylation of these peptides by CaM kinase I and CaM kinase IV was carried out for 5 min at 30 °C and a 50-μl reaction containing 50 mM PIPES, pH 7.5, 1 mM MgCl₂, 50 μM [γ-32P]ATP (2.5 Ci/mmol), and 50 μM peptide. Reactions were initiated by the addition of kinase and terminated by the addition of 10 μl of 30% trichloroacetic acid. Reaction mixtures were then spotted onto F-81 phosphocellulose paper (Whatman), washed extensively with water, and then counted for Cerenkov radiation.

**Protein Phosphorylation—α₉-CaM kinase II, made kinase-inactive with a K42M point mutation (referred to as α₉-CaM kinase II), as well as α₉-CaM kinase II with the S332A point mutation were expressed in COS-7 cells and purified as described (25, 31, 32). The reactions were then analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining of the CaM kinase II samples (data not shown). The concentration of CaM kinase II used between experiments ranged from 0.01 to 0.02 mg/ml. The phosphorylation reactions were initiated by adding either CaM kinase I or CaM kinase IV at a concentration of 5 μg/ml at the reaction mix and terminated by the addition of 5× Laemmli sample buffer. The reactions were then analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Scanning densitometry was performed on an Arcus AGFA II scanner using NIH Image for data analysis. Phosphorylation of α₉-CaM kinase II, by either CaM kinase I or CaM kinase IV was quantified by densitometry and assigned the value of 100%; phosphorylation of α₉-CaM kinase II, S332A was divided by this value to determine its corresponding percentage of phosphorylation.

**NLS Receptor Binding Experiments—**The NLS receptor (NLS-R) monopendulin fused with a His₈ tag within the bacterial expression vector pET-30a (Novagen) was a generous gift of S. Adam (Northwestern University, Chicago, IL) (33). The NLS-R construct was transferred into BL21 bacteria (Novagen) and grown in 200 ml of LB medium containing 50 μg/ml kanamycin and amplified with 1 mM isopropyl β-D-thiogalactopyranoside as described (33). The bacteria were pelleted, and the pellet was resuspended in 10 ml of 10% glycerol, 0.2% Nonidet P-40, 0.1 mM Tris (carboxyethylphosphine, 1 μM/ml leupeptin, 1 μM/ml aprotin, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium metabisulfite and 1 μg/ml pepstatin A. The bacterial suspension was frozen with liquid nitrogen, thawed, and then sonicated for a total of 3 min on ice at a setting of 50 W. The lysate was then centrifuged for 30 min at 14,000 × g at 4 °C. The resulting supernatant was then filtered through a 0.45-μM filter, aliquoted, quick frozen with liquid N₂, and then stored at −80 °C. The His₈-NLS-R was complexed to Ni²⁺-containing agarose resin (Qiagen) at a ratio of 6 mg of NLS-R-lysating contain 50 μl of resin in a 1 ml of buffer containing 40 mM Hepes, pH 7.0, 150 mM KOAc, 2 mM MgOAc, 20 mM imidazole, 1 mM TCEP, 0.1% Tween 20, 0.1% casamino acids (Difco), 200 μM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM/ml leupeptin, and 1 μM/ml pepstatin A. The bacterial suspension was frozen with liquid nitrogen, thawed, and then sonicated for a total of 3 min on ice at a setting of 50 W. The lysate was then centrifuged for 30 min at 14,000 × g at 4 °C. The resulting supernatant was then filtered through a 0.45-μM filter, aliquoted, quick frozen with liquid N₂, and then stored at −80 °C. The His₈-NLS-R was complexed to Ni²⁺-containing agarose resin (Qiagen) at a ratio of 6 mg of NLS-R-lysating contain 50 μl of resin in a 1 ml of buffer containing 40 mM Hepes, pH 7.0, 150 mM KOAc, 2 mM MgOAc, 20 mM imidazole, 1 mM TCEP, 0.1% Tween 20, 0.1% casamino acids (Difco), 200 μM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM/ml leupeptin, and 1 μM/ml pepstatin A. The bacterial suspension was frozen with liquid nitrogen, thawed, and then sonicated for a total of 3 min on ice at a setting of 50 W. The lysate was then centrifuged for 30 min at 14,000 × g at 4 °C. The resulting supernatant was then filtered through a 0.45-μM filter, aliquoted, quick frozen with liquid N₂, and then stored at −80 °C. **RESULTS**

**Cotransfection with CaM Kinase I or IV Blocks Nuclear Targeting of α₉-CaM kinase II**—As we previously reported (12, 13), α₉-CaM kinase II, which lacks a functional NLS, localizes exclusively to the cytoplasm (Fig. 1A) when transfected into COS-7 cells, while α₉-CaM kinase II, which contains an NLS, localizes almost entirely to the cell nucleus (Fig. 1B and Table I). We next sought to determine whether the activation of any known signal transduction pathway could alter the localization of α₉-CaM kinase II to the nucleus. In order to do this, α₉-CaM kinase II was cotransfected into COS-7 cells with cDNA constructs encoding several different protein kinases: the catalytic subunit of protein kinase A and constitutively active truncation mutants of CaM kinase I, CaM kinase IV, protein kinase C, and the protein phosphatase calcineurin. After 48 h, the localization of CaM kinase II was determined by immunofluorescence using a monoclonal antibody that recognizes α-CaM kinase II isoforms. No effect on the nuclear localization of α₉-CaM kinase II was noted when cotransfected with protein kinase A, protein kinase C, or calcineurin (data not shown). However, when cotransfected with constitutive, Ca²⁺/calmodulin-independent mutants of CaM kinase I or CaM ki-
nase IV, the localization of $\alpha_\gamma$-CaM kinase II was almost completely cytoplasmic (Fig. 1, C and D, and Table I). In order to determine whether this effect was cell type specific, we performed similar experiments in PC12 cells (a rat pheochromocytoma cell line) and HEK-293 cells (a human kidney cell line) and found a similar blockade of $\alpha_\gamma$-CaM kinase II nuclear targeting by CaM kinase I and CaM kinase IV (data not shown). COS-7 cells transfected only with CaM kinase I or CaM kinase IV did not show any detectable immunofluorescence when stained with the CaM kinase II antibody, ruling out the possibility that antibody cross-reactivity with CaM kinase I or CaM kinase IV was the basis for this result (data not shown).

The effect of constitutive CaM kinase I and CaM kinase IV on nuclear targeting was seen with another nuclear isoform of CaM kinase II detected with the use of a different primary antibody. We tested the effect of these kinases on targeting of $\delta_\gamma$-CaM kinase II, which also contains a functional NLS and has been shown to target to the nucleus when transfected into COS-7 cells (12). In this experiment, using epitope-tagged $\delta_\gamma$-CaM kinase II and an anti-tag antibody for immunostaining, constitutively active CaM kinase I and CaM kinase IV were able to block nuclear targeting of $\delta_\gamma$-CaM kinase II as effectively as was seen with $\alpha_\gamma$-CaM kinase II (data not shown).

The Blockade of CaM Kinase II Nuclear Targeting Is Specific—In order to determine whether CaM kinase I and CaM kinase IV exert a specific block on nuclear targeting of CaM kinase II or instead block nuclear import in a general fashion, we cotransfected constitutive CaM kinase I or CaM kinase IV into COS-7 cells with fos-lacZ, a fusion of the transcription factor fos with lacZ ($\beta$-galactosidase) that localizes to the nucleus as a result of a bipartite NLS derived from the fos protein (36). We found that nuclear localization of the fos-lacZ fusion protein was preserved when cotransfected into COS-7 cells under conditions identical to those in the experiments involving blockade of CaM kinase II nuclear targeting (data not shown). This suggests that CaM kinase I and CaM kinase IV exert a specific blockade on nuclear localization of CaM kinase II rather than a generalized blockade of nuclear import.

The First Ser following the NLS Is Required for Blockade of Nuclear Targeting—Blockade of nuclear import by phosphorylation of residues adjacent to an NLS has been seen for some but not all nuclear targeted proteins (Table II; reviewed in Ref. 24). We therefore sought to determine whether nuclear targeted isoforms of CaM kinase II have any possible phosphorylation sites adjacent to the NLS. As can be seen in Fig. 2, both $\alpha_\gamma$-CaM kinase II and $\delta_\gamma$-CaM kinase II, which have been shown to be targeted to the nucleus, as well as $\gamma_\gamma$-CaM kinase II, which has an identical NLS, all have a string of four Ser residues immediately following the core NLS sequence KKKR. These four Ser residues correspond to residues 332–335 in both $\alpha_\gamma$-CaM kinase II and $\delta_\gamma$-CaM kinase II and to residues 355–358 in $\gamma_\gamma$-CaM kinase II. In order to determine whether phosphorylation of one or more of these Ser residues is responsible for blockade in nuclear targeting by CaM kinase I and CaM kinase IV, we mutated all four Ser residues to Ala (S332–335A) in $\alpha_\gamma$-CaM kinase II. Like wild-type $\alpha_\gamma$-CaM kinase II, this mutant localizes almost entirely to the nucleus when transfected into COS-7 cells. Unlike $\alpha_\gamma$-CaM kinase II, however, nuclear targeting of this quadruple Ser to Ala mutant is not blocked by cotransfection with constitutive CaM kinase I or CaM kinase IV (Fig. 3 and Table I). To determine if only one Ser is responsible for this effect, we next mutated each of the four Ser residues individually to Ala. The mutant in which the first Ser following the NLS was mutated to Ala (S332A) shows nuclear targeting that is not affected by coexpression with CaM kinase I or CaM kinase IV and is indistinguishable from the targeting of the mutant with all four Ser residues mutated to Ala. In contrast, the other three mutants with single Ser to Ala mutations showed more efficient nuclear targeting of the kinase, we generated a mutant of CaM kinase II S332A and S333A mutants with and without CaM kinase I and CaM kinase IV. Immunostaining of the $\alpha_\gamma$-CaM kinase II S332A and S333A mutants with and without CaM kinase I or CaM kinase IV is shown in Fig. 3, and compiled data for all mutants are displayed in Table I.

A Negative Charge following the NLS Is Sufficient for Blockade of Nuclear Targeting—The preceding experiments clearly show that the Ser immediately following the CaM kinase II NLS is essential for blockade of nuclear targeting by CaM kinase I and CaM kinase IV and might be a target for phosphorylation by these kinases. To determine if a negative charge adjacent to the NLS of CaM kinase II is sufficient to block nuclear targeting of the kinase, we generated a mutant of $\alpha_\gamma$-CaM kinase II in which all four Ser residues following the NLS were mutated to Asp (S332–335D) to mimic the negative charge that phosphorylation of these residues would produce.

---

**Table I**

**Nuclear localization of CaM kinase II mutants coexpressed with CaM kinase I and CaM kinase IV**

Wild-type $\alpha_\gamma$-CaM kinase II as well as the listed Ser to Ala mutants were cotransfected into COS-7 cells with vector alone or with constitutively active mutants of CaM kinase I or CaM kinase IV. The localization of CaM kinase II within the cell nucleus or cytoplasm was determined by immunostaining as described under "Experimental Procedures" 48 h after transfection. 100 cells were counted in a blinded fashion for each combination listed, and the percentage of transfected cells with predominantly nuclear staining is listed.

| CaM kinase II          | With vector | With CaM kinase I | With CaM kinase IV |
|------------------------|-------------|-------------------|-------------------|
| $\alpha_\gamma$ Wild type          | 98          | 9                 | 10                |
| $\alpha_\gamma$KKKAAAAA     | 96          | 98                | 92                |
| $\alpha_\gamma$KKKASSSS     | 97          | 97                | 95                |
| $\alpha_\gamma$KKRKSASS     | 89          | 2                 | 0                 |
| $\alpha_\gamma$KKRKSASS     | 96          | 12                | 9                 |
| $\alpha_\gamma$KKRKSASSA     | 95          | 8                 | 12                |

* Wild type $\alpha_\gamma$-CaM kinase II contains the NLS followed by four Ser residues (KKRKSASS). All mutants listed are identical to wild type except for Ala substitutions in residues 332–335, which are underlined.

**Table II**

**Examples of inhibition of NLS function by phosphorylation**

| NLS-containing protein | Kinasea | NLS/phosphorylation siteb |
|------------------------|---------|--------------------------|
| $\alpha_\gamma$-CaM kinase II | CaM kinase I/IV | KKKRKS<sub>332</sub> |
| Lamin B2 (57) | Protein kinase C | S<sub>332</sub>TKGRRRIE |
| Cofilin (58, 59) | CaM kinase II | S<sub>332</sub>TQEVKKRK |
| v-Jun' (60, 61) | Cdk2 | S<sub>332</sub>TQEVKKRK |
| SW15 (62, 63) | Cdk/CDC28 | KKYENVS<sub>366</sub>PRKGRPRKDKGTSVS<sub>366</sub> |

a Although these kinases have been shown to be capable of phosphorylating the relevant sites leading to inhibition of NLS function, other kinases may be involved in vivo.

b The single-letter amino acid codes are used. NLS sequences are underlined, and phosphorylation sites are in boldface type and numbered according to their residue number in the respective protein.

c Cell cycle-dependent regulation of v-Jun nuclear localization is mediated by phosphorylation of Ser<sup>246</sup>, while c-Jun possesses Cys<sup>246</sup> and is constitutively nuclear.
When transfected into COS-7 cells, this mutant localized to the cytoplasm, consistent with the hypothesis that phosphorylation of one or more of these residues could block nuclear targeting of CaM kinase II (data not shown). In order to further examine this effect, we individually mutated Ser\textsuperscript{332} and Ser\textsuperscript{333} to both Asp and Glu. Mutation of Ser\textsuperscript{332} to either Asp or Glu caused the kinase to be localized entirely to the cytoplasm when transfected into COS-7 cells (Fig. 4, A and B), while mutation of Ser\textsuperscript{333} to Asp or Glu resulted in mutants with entirely normal nuclear localization (Fig. 4, C and D). 100 cells from each mutant were scored blindly for nuclear or cytoplasmic localization of the kinase as described for the experiments in Table I, and all 100 of the cells transfected with the S332D or S332E mutant of \(\alpha_\text{B}-\text{CaM kinase II} \) were scored as having cytoplasmic localization of the kinase, while all 100 of the cells transfected with the S333D or S333E mutant were scored as having nuclear localized kinase. Thus, a negatively charged residue replacing the first Ser following the NLS abolishes nuclear targeting of \(\alpha_\text{B}-\text{CaM kinase II} \), while a negative charge at the second position does not appreciably alter nuclear targeting.

CaM Kinases I and IV Can Phosphorylate the Ser Adjacent to the NLS of CaM Kinase II in Vitro—A study by Miyano et al. (37), which did not address nuclear targeting, demonstrated that a peptide encompassing the NLS and four adjacent Ser residues of \(\gamma_\alpha\text{-CaM kinase II} \) was a substrate for CaM kinase IV and that this phosphorylation was on the first Ser. In order to determine if CaM kinase I could also phosphorylate this site, we synthesized peptides derived from \(\delta_\gamma\text{-CaM kinase II} \) with the amino acid sequence CGVKKRKSSSSSVQME (the S peptide) as well as an otherwise identical peptide with the first Ser mutated to Ala, CGVKKRKASSSSSVQME (the A peptide). These peptides correspond to residues 326–340 of \(\delta_\gamma\text{-CaM kinase II} \); the initial Cys on each peptide was engineered for coupling purposes unrelated to these experiments. In agreement with the previous study, we found that purified wild type CaM kinase IV did phosphorylate the Ser-containing peptide in the presence of Ca\textsuperscript{2+} and calmodulin and that phosphorylation of the A peptide was more than 7-fold reduced compared with the S peptide. Additionally, we found that purified wild type CaM kinase I was also capable of phosphorylating the S peptide in the presence of Ca\textsuperscript{2+} and calmodulin and that phosphorylation of the A peptide was reduced by more than 4-fold compared with the S peptide (data not shown).

The ability of a kinase to phosphorylate a peptide does not ensure that the kinase will be able to phosphorylate that sequence within the structure of a large protein, however, and so we next sought to determine if CaM kinase I and CaM kinase IV could phosphorylate \(\alpha_\text{B}-\text{CaM kinase II} \) protein on this key Ser. We purified kinase-inactive mutants of \(\alpha_\text{B}-\text{CaM kinase II} \) and \(\alpha_\text{B}-\text{CaM kinase II} \) S332A (to avoid interference by auto-phosphorylation, \(\alpha_\text{B}-\text{CaMK II} \) and the S332A mutant were made kinase-inactive with a K42M mutation within the catalytic domain referred to as \(\alpha_\text{B}-\text{CaMK II} \)). We found that both purified wild-type CaM kinase I and CaM kinase IV were able to phosphorylate purified \(\alpha_\text{B}-\text{CaMK II} \), in the presence of Ca\textsuperscript{2+} and calmodulin, and that the level of phosphorylation was much less with the S332A mutant (Fig. 5B). Quantification by densitometry revealed that the level of phosphorylation of the S332A mutant of \(\alpha_\text{B}-\text{CaM kinase II} \) by CaM kinase I was 24.5 ± 3.6%, and the level of phosphorylation of this mutant by CaM kinase IV was 8.45 ± 4.99% as compared with the respective phosphorylation levels of \(\alpha_\text{B}-\text{CaM kinase II} \), containing Ser\textsuperscript{332}, which were assigned the value of 100% (Fig. 5C). Omission of either CaM kinase I or CaM kinase IV or the inactive CaM kinase II substrate resulted in no detectable phosphorylated band at this position (data not shown).

Blockade of Nuclear Targeting Is Not Dependent on the Position of the NLS within CaM Kinase II—We sought to deter-
mine whether moving the NLS of CaM kinase II from the central region of the protein (based on primary structure) to the N terminus would alter the ability of CaM kinase I or CaM kinase IV to block nuclear targeting of the kinase. To accomplish this, we generated a mutant of α-CaM kinase II containing an N-terminal fusion of 13 amino acids derived from the NLS region of α-CaM kinase II encompassing the KKRR sequence as well as the four adjacent serines and neighboring residues that complete the CaM kinase I and CaM kinase IV consensus phosphorylation sequences (Figs. 5A and 6A). When transfected into COS-7 cells, the NLS was able to function at the N terminus of normally cytoplasmic α-CaM kinase II, efficiently directing this protein to the nucleus. When cotransfected with constitutive CaM kinase I or CaM kinase IV, nuclear targeting of this protein was blocked as effectively as was found for wild type α-CaM kinase II (Fig. 6B).

A Negative Charge Adjacent to the NLS of CaM Kinase II Reduces Binding to an NLS Receptor—Does a negatively charged phosphate immediately adjacent to the positively charged NLS inhibit binding of this sequence to the NLS-R? To address this question, we developed an assay to assess binding of CaM kinase II to the NLS-R m-pendulin coupled via a His6 tag to Ni2+-complexed agarose resin. Other putative NLS receptor proteins including His6-tagged NPI-1, GST-tagged NPI-1, GST-tagged NPI-3, and GST-tagged karyopherin α were used in similar resin binding experiments, but none bound to α-CaM kinase II nearly as well as m-pendulin (data not shown), and so His6 m-pendulin was used for all subsequent NLS-R binding experiments. The NLS-R effectively bound NLS-containing α-CaM kinase II wild-type but showed almost no binding to NLS-deficient α-CaM kinase II wild-type, thus demonstrating the specificity of the assay for NLS-containing proteins (Fig. 7, A and B). In addition, a previously described mutant of δ-CaM kinase II in which the first two Lys residues of the NLS sequence were mutated to Asn (KKRK mutated to NNRK), disrupting nuclear targeting of the kinase (12), was similarly unable to bind to the NLS-R in this assay, while wild type δ-CaM kinase II bound as well to the NLS-R as did α-CaM kinase II (data not shown). Mutation of S332A in α-CaM kinase II did not inhibit binding to the NLS-R, but the S332D and S332E mutants demonstrated substantially reduced binding to the NLS-R (Fig. 7A). The S332D mutant of α-CaM kinase II did not show reduced binding to the NLS-R compared with wild-type α-CaM kinase II (data not shown). Sufficient experimental trials were performed to allow statistical comparison of the binding of α-CaM kinase II wild-type and the mutants. The data indicate that the S332A mutant binds to the NLS-R with high affinity, whereas the S332D and S332E mutants bind with significantly lower affinity (Table I).
and the four residues remaining from the hemagglutinin tag are boxed; the NLS sequence is highlighted in boldface type, the four residues remaining from the hemagglutinin tag are underlined, and the α-CaM kinase II sequence continues in its entirety, beginning with residue 4 of the wild type protein. This construct was transfected into COS-7 cells (B) in the presence of vector alone (left) or constitutive mutants of CaM kinase I (middle) or CaM kinase IV (right) as indicated, and CaM kinase II localization was visualized with immunofluorescence as described for Fig. 1.

tical analysis comparing the binding to the NLS-R of wild-type αCaM kinase II (standardized to 100% binding) versus that of α-CaM kinase II wild type and of αCaM kinase II mutants S332A and S332D. There was negligible binding (4.2 ± 4.6%) of α-CaM kinase II to the NLS-R, while the binding of the S332D mutant of αCaM kinase II was 51.9 ± 5.7% of αCaM kinase II wild type, and the binding of the S333A mutant was not significantly different (115 ± 16%) from αCaM kinase II wild type (Fig. 7B). These results demonstrate that a negatively charged residue immediately downstream from the NLS of αCaM kinase II reduces binding of this protein to the NLS-R compared with wild-type αCaM kinase II.

DISCUSSION

This paper demonstrates that phosphorylation by CaM kinase I or CaM kinase IV of a Ser adjacent to the NLS of nuclear targeted isoforms of CaM kinase II can block its nuclear targeting. CaM kinases I and IV have widespread tissue distribution and have been detected in such locations as the thalamus, hypothalamus, and heart, where nuclear targeted isoforms of CaM kinase II are expressed (10, 13, 38, 39). Thus, the colocalization of CaM kinase I or IV with nuclear targeted CaM kinase II that we have produced in situ through transfection is likely to occur in vivo. We have also examined the effects on nuclear targeting of CaM kinase II when coexpressed with protein kinase A or protein kinase C, two other multifunctional protein kinases, as well as the protein phosphatase calcineurin, all three of which have been shown to alter targeting of certain other NLS-containing proteins (reviewed in Ref. 24). None of these three signal transduction mediators was able to appreciably alter nuclear targeting of CaM kinase II. CaM kinase I and CaM kinase IV are both capable of modifying the function of many proteins through phosphorylation, and this study adds another important potential function to the cellular effects ascribed to these kinases.

The block of nuclear targeting with constitutively active truncation mutants of CaM kinases I and IV summarized above was not observed with full-length, Ca2+/calmodulin-dependent CaM kinase I or IV, even when the CaM kinase I T177D mutant was used or when CaM kinases I and IV were cotransfected with calmodulin and/or CaM kinase kinase and combined with varying lengths of stimulation with Ca2+/calmodulin (data not shown). Wild type CaM kinases I and IV stimulated with Ca2+/calmodulin were able to phosphorylate this key site in vitro (Fig. 5), however. Furthermore, phosho-
rylation of this site is likely to be sufficient to block nuclear targeting of CaM kinase II as mutant αCaM kinase II S332D and S332E are both completely cytoplasmic (Fig. 4). Activity-dependent modulation of CaM kinase II localization by phosphorylation in vivo may occur rather slowly during synthesis of nascent kinase or during cell division when the nuclear membrane is disrupted rather than as a rapid Ca\(^{2+}\)-dependent shuttle for the kinase in and out of the nucleus. It may be difficult to recreate in situ the conditions that govern regulation of CaM kinase II nuclear targeting in vivo.

There are a number of possible factors that may hinder the ability of wild type CaM kinases I and IV coupled with Ca\(^{2+}\)-elevating stimuli to effectively phosphorylate this site on CaM kinase II and block its nuclear targeting in the transfection system that we have used. The nature of our transfection experiments may require sustained phosphorylation of this site for as long as 48 h in order to see a block in nuclear targeting of the kinase. This is likely to occur with constitutive CaM kinase I or IV but may be difficult to achieve with wild type kinase. Activation of wild type CaM kinases I and IV in cultured cells is likely to be transient if it is like activation of CaM kinase II in these cells. In the case of CaM kinase II, we find that the in situ activation levels are low in the basal state and can be activated to higher levels only transiently. For example, the basal level of CaM kinase II autonomous activity (a measure of the activation state of the enzyme) is approximately 2% in PC12 cells (40, 41), and a similarly low basal level of CaM kinase II autonomy has been measured in COS-7 cells.\(^2\) In contrast, primary cultures of hippocampal neurons have a much higher basal level of CaM kinase II autonomy of 15.5% despite having a low resting [Ca\(^{2+}\)]\(_i\), of 15–43 nM (42), while homogenates of various brain regions have basal CaM kinase II autonomy values ranging from 4 to 10.5% (42, 43) and homogenates from cardiac muscle also have a relatively high basal level of CaM kinase II autonomy of approximately 8%.\(^3\) CaM kinase II autonomy can be increased by application of Ca\(^{2+}\)-elevating agents to cultured cells but then returns to basal levels over several minutes despite continued application of stimulus. Although equivalent activation experiments for CaM kinases I and IV have not been performed, these enzymes may also be less active in cultured cell lines than in primary neuronal or cardiac cells. This may make it difficult to maintain phosphorylation of the Ser adjacent to the CaM kinase II NLS for the 48 h required in our transfection experiments. Furthermore, activation of CaM kinases I and IV requires a complex series of phosphorylation events including both autophosphorylation and phosphorylation by an additional CaM kinase II/Fv kinase (28, 44–46), and it is possible that we are not able to fully activate wild type CaM kinase I and IV in our in situ cell system. The fact that neither cotransfection with CaM kinase with or without calmodulin nor the use of mutant CaM kinase I T177D allowed full-length CaM kinases I or IV to block nuclear targeting of CaM kinase II makes this somewhat less likely, however.

It is also possible that a kinase other than CaM kinase I or IV may actually phosphorylate this key site on CaM kinase II in vivo. The study by Miyano et al. (37) examining phosphorylation of this site on the peptide derived from the NLS of γCaM kinase II found that extracts from all brain regions examined could phosphorylate this Ser in a Ca\(^{2+}\)/calmodulin-dependent manner. It was not established whether this activity was the result of CaM kinase IV, CaM kinase I, or another kinase, although chromatography suggested that CaM kinase IV is the predominant Ca\(^{2+}\)/calmodulin-dependent kinase with this activity in cerebellum. Kinetic analysis demonstrated that this peptide is in fact only a moderately good substrate for CaM kinase IV, with \(K_m = 8.0 \mu M\) versus \(K_m = 2.6 \mu M\) for synapsin I and a similar \(V_{max}\) for both peptides (37). In vitro and in situ studies indicate the importance of the first Ser following the NLS for the regulation of nuclear targeting of CaM kinase II, and depending on the cell type, it may be CaM kinase I or IV or another as yet unidentified Ser/Thr kinase with similar specificity that phosphorylates this site in vivo.

Most NLS sequences can be moved within a protein and even fused onto completely different proteins and still retain their nuclear targeting function, although in some cases the position of an NLS relative to surrounding residues can have a profound impact on NLS function (47). We chose to move the NLS of αCaM kinase II from the central region of the protein’s primary structure to its extreme N terminus. Based on the “flower and petal” structure which has been proposed by Kanaseki et al. (48) to describe the quaternary holoenzyme structure of CaM kinase II, this would move the NLS a substantial distance from the central globular core to the peripheral catalytic region of the holoenzyme. We found the NLS to be able in this position to efficiently target the kinase to the nucleus. This N-terminal NLS mutant was designed to contain all of the CaM kinase I and CaM kinase IV consensus requirements contained within αCaM kinase II, and we found that both of these kinases were also capable of blocking nuclear localization of this mutant. This suggests that the effect of CaM kinases I and IV on nuclear targeting of CaM kinase II is a local effect on the region surrounding the NLS and probably does not depend on any particular three-dimensional structure of the NLS within the kinase, i.e. phosphorylation causing the kinase to fold in such a way as to obscure the NLS.

Alteration in nuclear targeting of proteins by phosphorylation is not a new phenomenon; there are many examples of phosphorylation either enhancing or inhibiting nuclear translocation of a variety of NLS-containing proteins (reviewed in Ref. 24). One mechanism for inhibition of NLS function is phosphorylation-induced NLS “masking,” which refers to the hypothesis that a phosphate adjacent to the NLS inhibits binding to the NLS-R as a result of its charge or a conformational effect (24). Given that negatively charged regions of the NLS-R are thought to bind to the positively charged NLS of nuclear targeted proteins for nuclear import to occur (49), it is not surprising that a negative charge immediately adjacent to this site may inhibit this interaction. Although this theory has been advanced to explain inhibition of nuclear targeting by phosphorylation of several NLS-containing proteins (Table II), this has never been demonstrated directly.

Using an NLS-R binding assay, we present evidence that the alternatively spliced 13-amino acid sequence containing the NLS consensus sequence KKKR is critical for binding of αCaM kinase II to the NLS-R m-pendulin. Additionally, we demonstrate that a negative charge adjacent to this NLS reduces binding to the NLS-R by approximately 50%, while mutation of this site to a neutral Ala does not significantly alter binding to the NLS-R. A recent study examining the enhancement of nuclear targeting of the SV40 large T antigen (a protein with multiple phosphorylation sites, some of which enhance while others inhibit nuclear targeting) by phosphorylation gave the first evidence that phosphorylation can affect binding of an NLS-containing protein to an NLS-R (50). In this study, the authors report that phosphorylation of SV40-LacZ fusions by casein kinase II, which causes a large increase in nuclear import rate, may be mediated by an observed 40% increase in binding of this protein to the NLS-R. Thus, the 50%
decrease in NLS-R binding that we have observed with a negatively charged residue adjacent to the NLS appears to be in the range that can have a profound impact on nuclear targeting. Perhaps a doubly charged phosphate adjacent to the NLS will lead to an even larger decrease in NLS-R binding than we report here, but the stoichiometric phosphorylation of CaM kinase II by CaM kinase I or CaM kinase IV that will be required to examine this may be difficult to attain in vitro. Regardless, our mutational analysis demonstrates that a single negatively charged residue is able to completely block nuclear targeting; whether this is wholly or only partly mediated by the 50% decrease in binding to the NLS-R that we have observed has not been established.

The possibility that there is cross-talk within the family of multifunctional Ca2+/calmodulin-dependent protein kinases raises some very intriguing questions. It is known that different frequencies of Ca2+ stimuli can lead to completely opposite effects on cellular phenotype; for example, high frequency stimulation of neurons leads to long term synaptic potentiation, while the same stimulus delivered at lower frequencies causes long term synaptic depression (51). Furthermore, several studies have implicated CaM kinase II in the generation of long term synaptic potentiation (52, 53), which also requires new gene transcription in order to be maintained (54). The finding that CaM kinase II is capable of repressing activity of the transcription factors cyclic AMP response element-binding protein and ATF-1 while CaM kinase I and CaM kinase IV both increase gene transcription in order to be maintained (54). The finding that CaM kinase II is capable of repressing activity of the transcription factors cyclic AMP response element-binding protein and ATF-1 while CaM kinase I and CaM kinase IV both increase gene transcription in order to be maintained (54). The finding that CaM kinase II is capable of repressing activity of the transcription factors cyclic AMP response element-binding protein and ATF-1 while CaM kinase I and CaM kinase IV both increase gene transcription in order to be maintained (54). The finding that CaM kinase II is capable of repressing activity of the transcription factors cyclic AMP response element-binding protein and ATF-1 while CaM kinase I and CaM kinase IV both increase gene transcription in order to be maintained (54).