Cytoplasmic localization of calcium/calmodulin-dependent protein kinase I-α depends on a nuclear export signal in its regulatory domain

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Abstract Calcium/calmodulin-dependent protein kinase I-α (CaMKI-α) is a ubiquitous cytosolic enzyme that phosphorylates a number of nuclear proteins in vitro and has been implicated in transcriptional regulation. We report that cytoplasmic localization of CaMKI-α depends on CRM1-mediated nuclear export mediated through a Rev-like nuclear export signal in the CaMKI-α regulatory domain. Interaction of CaMKI-α with a CRM1 complex in vitro is enhanced by incubation with calcium/calmodulin. Translocation of CaMKI-α into the nucleus involves a conserved sequence located within the catalytic core. Mutation of this sequence partially blocks nuclear entry of an export-impaired mutant of CaMKI-α.

Keywords: CRM1; Leptomycin B; PC12 cell; GFP; Nuclear export; Calcium/calmodulin-dependent protein kinase I

1. Introduction

Calcium/calmodulin-dependent protein kinase I (CaMKI) has been implicated in several cellular processes including embryonic development [1], cell division [2–4], hormone production [5], neurite outgrowth [6], myogenesis and actin filament organization [7,8] and transcriptional activation [9,10]. A number of substrates have been identified for CaMKI including synapsin I and II [11], the cystic fibrosis transmembrane conductance regulator [12], myosin II regulatory light chain kinase [8], cAMP response element-binding protein (CREB) [13], activating transcription factor (ATF-1) [14], histone deacetylases 4 and 5 [7], and the translation initiation factor eIF4GII [10]. Yet, the physiological role of CaMKI is not well understood.

Multiple isoforms of CaMKI have been identified with distinct subcellular and tissue distributions. CaMKI-α has a broad tissue distribution and is primarily cytosolic, although nuclear localization of CaMKI-α has been suggested in a subset of neurons [15,16]. CaMKI-β1 and -β2 localize to both the cytoplasm and nucleus, and are found in liver, heart, lung, kidney, spleen and testes (β1), and brain (β2) [17,18]. CaMKI-γ1 and -γ2 appear to be anchored to Golgi and plasma membranes in neurons [19,20]. Recently, a sixth isoform (CaMKI-δ) has also been identified [21]. The tissue distribution and subcellular localization of CaMKI isoforms are likely to be critical in determining their physiological roles. Since calmodulin (CaM) kinases have been proposed to play a role in nuclear processes [9,10], the regulation of nuclear trafficking of CaMKI isoforms is of interest.

Here, we report the identification of a nuclear export signal (NES) in the regulatory domain of CaMKI-α and demonstrate that nuclear export is mediated through CRM1. Association of CaMKI-α with a CRM1 complex is enhanced in the presence of calcium (Ca^{2+})/CaM, suggesting that activation of the kinase may facilitate its nuclear export. We also report identification of an amino acid sequence ($K^{293}R^{294}$) in the catalytic core of CaMKI-α that appears to play a role in nuclear import.

2. Materials and methods

2.1. Cell culture and transfection

PC12 cells were maintained on collagen (Upstate Biotechnologies) in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% horse serum/5% fetal bovine serum/100 μg/ml penicillin–streptomycin. For microscopic analysis, PC12 cells were plated on poly L-lysine- (Peptide Institute Inc.) and laminin-coated (Invitrogen) glass coverslips. Cos7 cells were maintained in DMEM supplemented with 10% fetal bovine serum/100 μg/ml penicillin–streptomycin. Transfections were performed using FuGene 6 (Roche).

2.2. Plasmid construction

GFP-fusion plasmids were generated by subcloning PCR-amplified full length CaMKI-α (CaMKI374) and CaMKI-α truncations (CaMKI362, CaMKI344, CaMKI322, CaMKI305, CaMKI296, and CaMKI293) into the *Bgl*II and *Kpn*I sites of pEGFPC-1 (Clontech). Mutations of full length and truncated CaMKI-α were generated using the QuickChange kit (Stratagene). pC1-HA-CRM1 and pQE32-RanQ69L [22,23] were gifts from Dr. Mark Hannink (University of Missouri).

2.3. Subcellular localization of CaMKI-α

Transfected PC12 cells were examined by live cell fluorescence microscopy after the following treatments: 2 h incubation with 200 ng/ml leptomycin B (LMB; Sigma), 30 min with 2 mM $CaCl_2$/1 mM $MgCl_2$ and either 40 mM KCl or 4 μM ionomycin (Molecular Probes), 30 min with 10 μM KN62 (Calbiochem), or 1 h with 10 μM BAPTA-AM (Molecular Probes). Endogenous CaMKI-α was examined by immunofluorescence

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Abbreviations: CaMK, calcium/calmodulin-dependent protein kinase; CaM, calmodulin; Ca^{2+}, calcium; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization signal

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after treatment with KCl, ionomycin, KN62, or BAPTA-AM with or without pretreatment with 200 ng/ml LMB. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated for 1 h with 1:500 CC77 antibody [16] followed by 30 min incubation with 1:1000 Alexa Fluor anti-rabbit antibody (Molecular Probes). Images were captured at 400× magnification.

The cytoplasmic and nuclear density of GFP fluorescence within individual cells was determined using Image J software (NIH). The total number of pixels was calculated for an equivalent area in both cytoplasm and nucleus (corrected for background fluorescence) for each cell examined. The resulting values were used to calculate the cytoplasmic:nuclear pixel density ratio (C:N) for each cell in an experimental group. An experimental group consisted of a minimum of five cells from the same transfection or 15 cells per condition in immunofluorescence experiments. The mean C:N ratios from three experimental groups (≥15 cells total) were averaged to determine the mean ratio for each expressed protein.

2.4. In vitro binding assay

The protocol for in vitro binding of GST-CaMKI-α to HA-CRM1 was modified from [22]. Crude lysate from Cos7 cells expressing HA-CRM1 was incubated for 2 h at 4 °C with anti-HA affinity matrix (Covance). The HA-CRM1-bound matrix was washed, divided evenly into three tubes, and incubated overnight with GST-CaMKI-α (with or without a 10 min preincubation with 2 mM $CaCl_2$ and 20 μg/ml CaM (Sigma)) in the absence or presence of His-RAN-GTP. After washing, samples were eluted with SDS–PAGE sample buffer.

2.5. SDS–PAGE and Western-blot analysis

Samples were separated using SDS–PAGE (10% acrylamide) and transferred to nitrocellulose. HA-CRM1 was detected with mouse monoclonal anti-HA antibody (Covance, 1:200). GST-CaMKI-α was detected using rabbit polyclonal anti-GST antibody (Santa Cruz, 1:2000). His-RAN was detected with a rabbit polyclonal Ran antibody (Covance, 1:2000). Secondary antibodies were diluted 1:2000.

2.6. Statistical analysis

Statistical analysis was by ANOVA followed by the Tukey HSD post hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. Subcellular distribution of CaMKI-α

Like the native protein [16], CaMKI-α, fused at its N-terminus to green fluorescent protein (GFP-CaMKI374), localized to the cytoplasm of transfected cells. In contrast, CaMKI-α truncated to amino acid 293 (GFP-CaMKI293) was found throughout the nucleus and cytoplasm (Fig. 1A). To ascertain the effect of the autoinhibitory domain and C-terminal sequences on localization of CaMKI-α, a series of C-terminal truncations were generated and fused to GFP (Fig. 1B). Truncation to residue 362 (GFP-CaMKI362) removed a C-terminal proline-rich domain. Truncation to amino acids 344 and 322 (GFP-CaMKI344 and GFP-CaMKI322) left the autoinhibitory and CaM binding domains intact. Truncation to residue 305 (GFP-CaMKI305) deleted the CaM binding domain but not the autoinhibitory domain, resulting in a constitutively inactive enzyme [24]. Further truncation to amino acids 296 and 293 (GFP-CaMKI296 and GFP-CaMKI293) removed the autoinhibitory domain, generating CaM-independent, constitutively active forms of CaMKI-α [24].

Two distinct localization patterns were observed for CaMKI-α truncations. GFP-CaMKI374, -CaMKI362, -CaMKI344, and -CaMKI322 localized to the cytoplasm, whereas GFP-CaMKI305, -CaMKI296, and -CaMKI293 were distributed in both the cytoplasm and nucleus (Figs. 1A, C and D). These data suggested that the region between residues 305 and 322 is critical for nuclear exclusion of CaMKI-α.

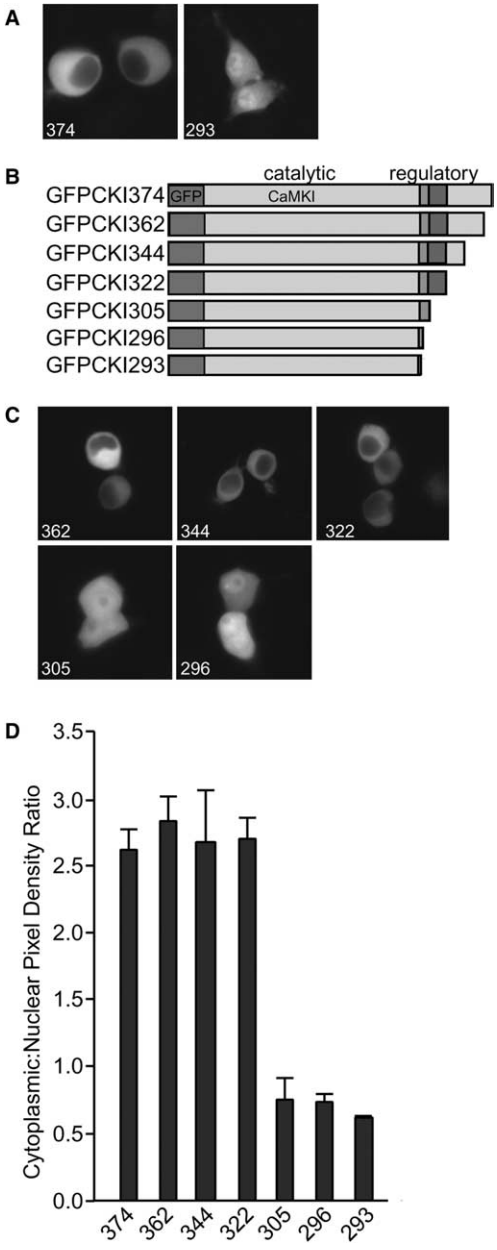

Fig. 1. Subcellular distribution of CaMKI-α. (A) Fluorescence images of cells expressing GFP-CaMKI374 (374) and GFP-CaMKI293 (293). (B) Schematic representation of C-terminal truncations of CaMKI-α. (C) Fluorescence images of cells expressing GFP-CaMKI362 (362), GFP-CaMKI344 (344), GFP-CaMKI322 (322), GFP-CaMKI305 (305), and GFP-CaMKI296 (296). (D) Cytoplasmic:nuclear ratios of GFP fluorescence in transfected cells. Error bars: ±S.D. ANOVA: $F(6, 115) = 69.59$. Post hoc tests revealed that C:N ratios of 374, 362, 344 and 322 were significantly different from those of 305, 296, 293 ($P < 0.001$).

3.2. CaMKI-α contains a NES and is exported by CRM1

Examination of residues 305–322 revealed a putative NES ($_{315}$VVRHMRKLQL$_{321}$) similar to the recognized consensus ($LX_{(1-3)}LX_{(2-3)}LXL$) of the Rev-like NES [25,26] (Fig. 2A). To determine whether this sequence serves as a functional NES,

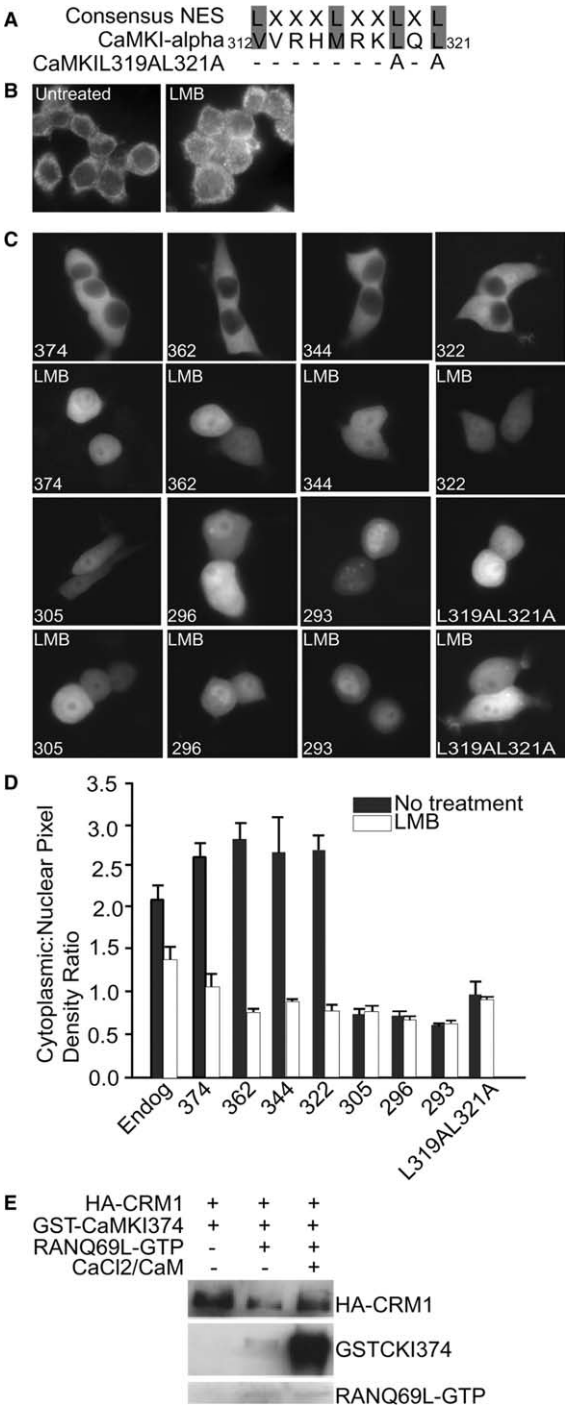

Fig. 2. Identification of a NES in CaMKI-α. (A) Comparison of consensus NES sequence with residues 312–321 in CaMKI-α. (B) Immunofluorescence images of endogenous CaMKI in the absence or presence of LMB. (C) Fluorescence images of cells expressing GFP-CaMKI-α proteins in the absence or presence of LMB. (D) Cytoplasmic:nuclear ratios of CaMKI distribution in the absence or presence of LMB. Abbreviations as in Fig. 1. Black: no treatment; white: 2 h incubation with 200 ng/ml LMB. Error bars: ±S.D. ANOVA: $F(7,252) = 62.11$. Post hoc tests revealed a significant effect of LMB treatment for endogenous CaMKI (Endog), 374, 362, 344, and 322 ($P < 0.001$); GFP-CAMKI^{L319A,L321A} (L319AL321A) was significantly different from 374 ($P < 0.001$). (E) Western-blot analysis of in vitro binding of GST-CaMKI-α to HA-CRM1 supporting the idea that there is an interaction of HA-CRM1, RAN-GTP and GST-CaMKI-α and showing the effect of preincubation with Ca^{2+}/CaM.

two leucine residues at positions 319 and 321 were mutated to alanine to generate GFP-CaMKI^{L319A,L321A}. The mutant protein displayed uniform cytoplasmic and nuclear localization within transfected cells (Figs. 2C and D).

Cells were treated with LMB, a fungicide that binds to and inhibits the export activity of CRM1. LMB treatment resulted in increased nuclear localization of endogenous CaMKI (Figs. 2B and D) and a uniform distribution of GFP-CaMKI374, -CaMKI362, -CaMKI344, and -CaMKI322 in the cytoplasm and nucleus of transfected cells (Figs. 2C and D), supporting the idea that CaMKI-α is exported from the nucleus in a CRM1-dependent manner. LMB treatment of cells expressing GFP-CaMKI305, -CaMKI296, -CaMKI293, or -CaMKI^{L319A,L321A} did not alter localization of these proteins, suggesting that the NES located between residues 315 and 321 is responsible for the cytoplasmic localization of CaMKI-α.

The ability of CaMKI-α to interact with CRM1 was examined in vitro. GST-CaMKI-α was incubated with HA-tagged CRM1 bound to an anti-HA affinity matrix in the presence or absence of RAN-GTP (a requisite cofactor for binding of CRM1 to NES-containing proteins) and Ca^{2+}/CaM. GST-CaMKI-α did not bind CRM1 in the absence of RAN-GTP (Fig. 2E). Preincubation of GST-CaMKI-α with Ca^{2+}/CaM increased binding of GST-CaMKI-α to CRM1 (Fig. 2E), suggesting that activation of CaMKI-α would facilitate its export from the nucleus. Although an equal volume of HA-CRM1 bound matrix was used for each experimental condition, variations in the amount of HA-CRM1 eluted from the matrix were apparent (Fig. 2E). The increase in GST-CaMKI-α bound to the matrix in the presence of Ca^{2+}/CaM are seen despite variations in HA-CRM1.

3.3. Identification of a basic amino acid sequence involved in CaMKI-α nuclear import

Three basic amino acid motifs ($K^{40}R^{41}$, $K^{53}K^{54}$, and $K^{263}R^{264}$) were mutated to alanine. These mutations were made on the GFP-CaMKI^{L319A,L321A} background, since only low levels of CaMKI are observed in the nucleus without these mutations. While neither GFP-CaMKI^{L319A,L321A,K40A,R41A} nor GFP-CaMKI^{L319A,L321A,K53A,K54A} displayed an altered distribution, GFP-CaMKI^{L319A,L321A,K263A,R264A}was partially, but significantly, excluded from the nucleus (Figs. 3A and B), suggesting that $K^{263}R^{264}$ may be involved in nuclear import of CaMKI-α.

3.4. Kinase activity and nuclear export

To determine the subcellular distribution of catalytically inactive forms of CaMKI-α, the localization patterns of GFP-CaMKI374^{K49A} and GFP-CaMKI293^{K49A} were examined. Residue K^{49} is required for kinase activity and mutation of this residue to alanine results in a catalytically inactive kinase [27]. This mutation did not alter subcellular distribution of either form of CaMKI-α (Figs. 4A and B), suggesting that activity alone is not enough to determine its localization.

In vitro analysis showed that the amount of CaMKI-α associated with CRM1 increased in the presence of Ca^{2+}/CaM. To test whether kinase activation might lead to increased export in cell culture, the subcellular localization of GFP-CaMKI374 (in transfected cells) or endogenous CaMKI-α was examined in cells treated with KCl or ionomycin (Figs. 4C and D). No difference in subcellular distribution

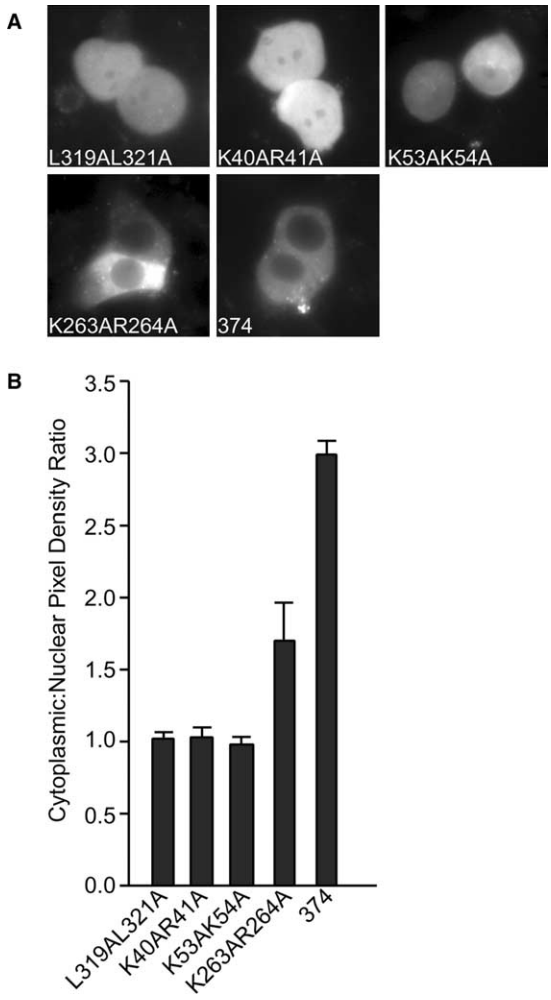

Fig. 3. Identification of a basic amino acid sequence involved in CaMKI-α nuclear translocation. (A) Fluorescence images of cells expressing GFP-CaMKI^{L319A,L321A} (L319AL321A), CaMKI^{L319A,L321A,K40A,R41A} (K40AR41A), CaMKI^{L319A,L321A,K53A,K54A} (K53AK54A), CaMKI^{L319A,L321A,K263A,R264A} (K263AR264A), and GFP-CaMKI374 (374). (B) C:N ratios of GFP fluorescence in transfected cells. Error bars: ±S.D. ANOVA: $F(4, 87) = 50.74$. Post hoc test revealed that L319AL321A, K40AR41A, and K53AK54A were significantly different from 374 and K263AR264A ($P < 0.004$). K263AR264A was significantly different from 374 ($P < 0.001$).

was detected; however, CaMKI-α is largely excluded from the nucleus in untreated cells, so an increase in nuclear export would not be easily detected. Treatment with the intracellular calcium chelator BAPTA-AM resulted in a very small but significant decrease in C:N ratios in both endogenous CaMKI and transfected GFP-CaMKI374 compared to untreated cells, and the CaM kinase inhibitor KN62 slightly decreased C:N ratios for GFP-CaMKI374, although it does not appear that these agents substantially inhibit nuclear export of CaMKI-α (Figs. 4C and D). Similar experiments in transfected cells expressing GFP-CaMKI^{L319A,L321A} (Figs. 4C and D), or when endogenous CaMKI was examined in untransfected cells preincubated with LMB (not shown), showed no changes in CaMKI-α localization. These results suggest that nuclear import is not significantly regulated by kinase activation.

4. Discussion

The best characterized NES are short leucine-rich peptides containing 4–5 hydrophobic residues. Mutational analyses of NES have shown that the hydrophobic residues, particularly leucines in the C-terminal portion of the NES, are required for nuclear export [25,26]. We have identified a NES in CaMKI-α with the sequence ^{312}VVRHMRKLQL^{321}. Mutation of leucines 319 and 321 to alanine resulted in uniform distribution of the kinase in the nucleus and cytoplasm, suggesting that these residues are critical for nuclear export of CaMKI-α.

Export of NES-containing proteins is mediated primarily through CRM1, which binds to NES-cargoes cooperatively with RAN-GTP. After transport to the cytoplasm, hydrolysis of RAN-GTP causes dissociation of the trimer and release of the cargo [28,29]. LMB inhibits CRM1-mediated export by binding to CRM1 and preventing its interaction with NES-cargo. LMB treatment of cells expressing GFP-CaMKI-α resulted in uniform distribution of CaMKI-α. Thus, nuclear export of CaMKI-α is likely mediated through CRM1.

The CaM-binding domain of CaMKI-α extends from amino acids 294 to 315 and might partially overlap the NES. For this reason, we initially hypothesized that CRM1 and CaM might compete for binding to CaMKI-α. Instead, binding of CaMKI-α to CRM1 in vitro was enhanced in the presence of Ca^{2+}/CaM, suggesting that CaM and CRM1 may have non-overlapping binding sites. These results are likely explained by conformational changes that occur upon binding of Ca^{2+}/CaM to CaMKI-α. In the absence of Ca^{2+}/CaM, the CaM-binding domain interacts with the catalytic domain and the NES may be less accessible [30,31]. Upon CaM binding, the conformational changes that remove the regulatory domain from the catalytic site potentially make the NES more accessible to CRM1, thus facilitating nuclear export of activated CaMKI-α.

Previous studies demonstrated that truncated, constitutively active CaMKI could phosphorylate nuclear proteins in neuronal cultures [14], suggesting that activation might play a role in nuclear access; however, in these previous studies, truncating CaMKI to generate the constitutively active form deleted the NES. The current study demonstrates that activity alone cannot account for the localization of CaMKI-α, since inactivation of the kinase by mutation of K^{49} or activation with KCl or ionomycin did not significantly affect localization. This contrasts with recent studies of CaMKIV, whose catalytic activity is needed for nuclear entry [32].

A comparison of the regulatory domains of CaMKI-α with other isoforms of CaMKI or CaMKIV shows that cytosolic CaMKI-γ/CKLiK [19,20,33] and CaMKI-δ [21] also contain leucine-rich, NES-like sequences in their regulatory domains (Fig. 5). In contrast, this domain is not conserved in CaMKIV, which is known to localize to the nucleus in many neuronal cell types [34]. CaMKI-β, which has a relatively uniform cellular distribution, has a glutamine in place of the C-terminal leucine within this domain [17] (Fig. 5). It is not yet known whether this single residue change could account for the localization pattern of CaMKI-β.

Classical nuclear localization signals (NLS) are lysine and arginine-rich motifs arranged as a single stretch (for example, the PKKKRKV NLS of the SV40 large T antigen) or as two short stretches separated by a flexible region (such as the bi-

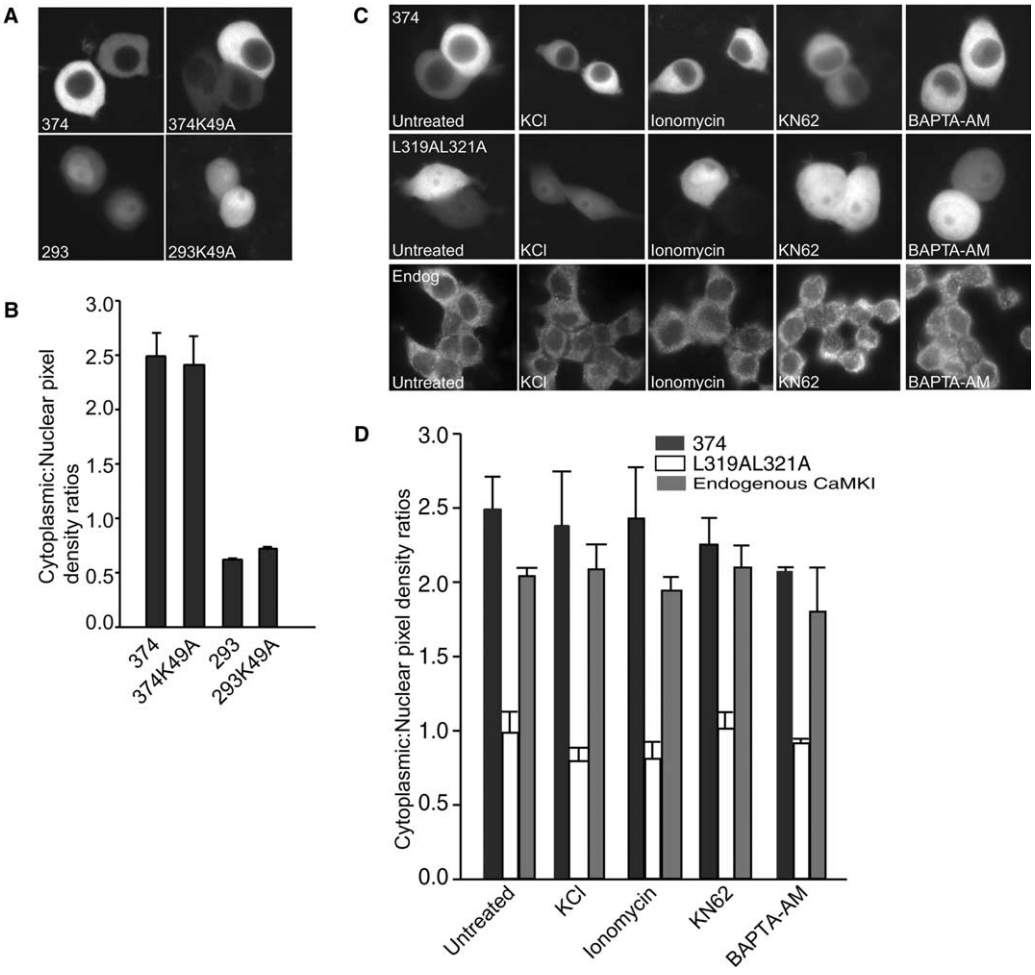

Fig. 4. Role of kinase activity in nucleocytoplasmic transport. (A) Fluorescence images of cells expressing GFP-CaMKI374 (374), GFP-CaMKI374^{K49A} (374K49A), GFP-CaMKI293 (293), and GFP-CaMKI293^{K49A} (293K49A). (B) C:N ratios of GFP fluorescence in transfected cells. Error bars: ±S.D. ANOVA: $F(3, 87) = 110.91$ (no significant effect of K49A mutation). (C) Fluorescence images of cells expressing GFP-CaMKI374 or GFP-CaMKI^{L319AL321A} and immunofluorescence images of endogenous CaMKI (Endog) in cells after no treatment or treatment with KCl, ionomycin, KN62 or BAPTA-AM. (D) C:N ratios of experimental conditions shown in C. Black (GFP-CaMKI374), white (GFP-CaMKI^{L319A,L321A}), and gray (endogenous). Error bars: ±S.D. ANOVA $F(9, 263) = 48.548$. Post hoc tests revealed a significant effect of treatment with BAPTA-AM or KN62 on the GFP-CaMKI374 construct ($P < 0.002$), and a significant effect of treatment with BAPTA-AM on endogenous CaMKI ($P < 0.01$).

partite NLS of nucleoplasmin, KRPAATKKAGQAKKK) [35]. Examination of the primary sequence of CaMKI-α revealed a stretch of basic amino acids extending from residues 298 to 307. Deletion of these residues (CaMKI296, CaMKI293) did not block nuclear entry of CaMKI-α. A partial disruption in nuclear translocation of CaMKI-α resulted from mutation of residues $K^{263}R^{264}$, suggesting that these residues may comprise part of a NLS. These residues are conserved across the CaM kinase family and in protein kinase A [27] and may, therefore, have a functional role in the kinase. Import of NLS-cargo is mediated by importin-α, which binds to the NLS and then forms a complex with importin-β that carries the cargo through the nuclear pore [35]. CaMKI-α does not appear to bind to importin-α in vitro [32]. Thus, CaMKI-α may interact with other members of the nuclear import machinery or may enter the nucleus coupled to other NLS-cargo(s). Further analysis of nuclear import mutants of CaMKI-α is required to understand fully the mechanism by which CaMKI-α translocates to the nucleus.

Consensus NES	L	X	X	X	L	X	X	L	X	L
CaMKI-alpha	V	V	R	H	M	R	K	L	Q	L
CaMKI-beta	F	L	R	H	I	R	K	L	G	Q
CaMK-gamma	V	V	H	H	M	R	K	L	H	M
CaMKI-delta	V	V	R	H	M	R	K	L	H	L
CKLiK	V	V	R	H	M	R	K	L	H	L
CaMKIV	V	V	A	S	S	R	L	G	S	A

Fig. 5. Comparison of NES-like sequences of CaMK isoforms. Alignment of the consensus NES with the NES of CaMKI-α and the corresponding sequence of CaMKI-α, -β, -γ, and -δ and CaMKIV. Conserved leucines within the consensus sequence and corresponding amino acids in CaMK isoforms are highlighted with gray boxes.

These studies indicate that the cytoplasmic localization of CaMKI-α is highly dependent on efficient CRM1-mediated nuclear export. Furthermore, the in vitro results showing an increased interaction of CaMKI-α with the CRM1 complex in the presence of Ca^{2+}/CaM suggest that activation of the kinase may enhance the efficiency of nuclear export. Thus, multiple cellular processes are required to maintain the steady-state

localization of CaMKI-α in the cytosol and not in the nucleus. This implies that cytosolic substrates of CaMKI-α are likely the most physiologically relevant. Notably, the best characterized substrates for CaMKI-α are cytosolic, including synapsin I and II [11], the cystic fibrosis transmembrane conductance regulator [12], myosin II regulatory light chain kinase [8], and the translation initiation factor eIF4GII [10]. Other evidence suggests a role for cytoplasmic CaMKI in cell cleavage progression in *Xenopus* oocytes [36] and in actin filament reorganization in mammalian cells [8]. Recently, it has been reported that cytosolic CaMKI-α is involved in growth cone motility and axon outgrowth in neonatal cerebellar granule neurons [6], supporting a role for CaMKI-α in signal transduction at the cytoskeleton/plasma membrane interface.

In vitro, a number of nuclear substrates for full length CaMKI have been identified including CREB [13] and ATF-1 [14]; however, in vivo efficient export of CaMKI-α may also be important for restricting its access to nuclear substrates, especially transcription factors. Ca^{2+}/CaM kinase-dependent regulation of gene transcription may also be restricted to particular cell types. Expression of CaMKIV, a kinase with well-defined effects on transcription factor activity [34], is restricted to sub-populations of neurons [37], while CaMKI is ubiquitously expressed [16]. Although CaMKI-α is rarely detected in the nucleus, a role for the kinase in phosphorylation of nuclear substrates under some physiological circumstances cannot be completely ruled out.

Several studies examining the role of CaMKI-α have used the truncated, constitutively active form of the enzyme. This truncation lacks a NES and is therefore localized uniformly to both the cytoplasm and nucleus. In light of the data presented here, it may be necessary to revisit these studies, particularly those which implicate CaMKI-α in phosphorylation of nuclear substrates, to determine their physiological relevance.

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