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Importin α transports CaMKIV to the nucleus without utilizing importin β

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Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMKIV) plays an essential role in the transcriptional activation of cAMP response element-binding protein-mediated signaling pathways. Although CaMKIV is localized predominantly in the nucleus, the molecular mechanism of the nuclear import of CaMKIV has not been elucidated. We report here that importin α is able to carry CaMKIV into the nucleus without the need for importin β or any other soluble proteins in digitonin-permeabilized cells. An importin β binding-deficient mutant (ΔIBB) of importin α also carried CaMKIV into the nucleus, which strongly suggests that CaMKIV is transported in an importin β-independent manner. While CaMKIV directly interacted with the C-terminal region of importin α, the CaMKIV/importin α complex did not form a ternary complex with importin β, which explains the nonrequirement of importin β for the nuclear transport of CaMKIV. The cytoplasmic microinjection of importin α-ΔIBB enhanced the rate of nuclear translocation of CaMKIV *in vivo*. This is the first report to demonstrate definitely that mammalian importin α solely carries a cargo protein into the nucleus without utilizing the classical importin β-dependent transport system.

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Introduction

The import of macromolecules from the cytoplasmic compartment to the nucleus generally requires soluble transport receptors. The use of the classical basic type nuclear localization signal (cNLS) in conjunction with digitonin-permeabilized semi-intact cells enabled the molecular mechanisms of the classical nuclear protein import to be studied. Importin α (karyopherin α) recognizes a cargo protein containing cNLS, and the importin α is further recognized by importin β (karyopherin β) to form a nuclear pore-targeting complex (PTAC). This ternary complex interacts with the phenylalanine–glycine (FG) repeat domains of nucleoporins that are present in the nuclear pore complexes (NPCs), and the interaction is crucial for the passage of macromolecules. A small GTPase Ran that is predominantly GTP bound in the nucleus binds to importin β in the nucleoplasm to trigger the dissociation of the ternary complex. The nature of the dissociation of the cargo protein from importin α is unclear, but in yeast, it has been proposed that Nup2p, together with Cse1p (a yeast homolog of the cellular apoptosis susceptibility gene product, CAS) and RanGTP, assists the release of the cargo from importin α. The complexes of RanGTP/CAS/importin α and RanGTP/importin β are exported back to the cytoplasm where they are dissociated by the hydrolysis of GTP by Ran to complete the cycle of transport receptors (for reviews, see Görlich and Mattaj, 1996; Mattaj and Englmeier, 1998; Yoneda, 2000; Stewart *et al*, 2001).

It has also been shown that multiple classes of importin β-like molecules that are present in a cell carry a variety of transport cargoes into the nucleus. Transportin, one of the importin β family members, mediates the nuclear import of a specific signal, termed M9, which was identified in hnRNP A1; the M9-containing cargoes are recognized by transportin without the aid of adaptor molecules such as importin α (Pollard *et al*, 1996). It has also been demonstrated that importin β by itself is able to transport cargoes including cyclin B1, sterol regulatory element-binding protein-2 (SREBP-2) and Smad3 through direct binding (Moore *et al*, 1999; Nagoshi *et al*, 1999; Kurisaki *et al*, 2001). It has also recently been reported in an X-ray crystallography study that importin β uses characteristic long helices, analogous to a pair of chopsticks, to bind to the active form of SREBP-2 (Lee *et al*, 2003).

It is also well known that importin α functions as an adaptor molecule between the cNLS-containing cargo and importin β. Structural analyses of importin α revealed the existence of three functional domains: an importin β binding (IBB) domain in the N-terminus, a hydrophobic central domain known as the armadillo (arm) repeat domain and a short acidic domain in the C-terminus. A cNLS binds to two sites within the arm repeat domain, whereas the C-terminal acidic domain binds to CAS, which mediates the nuclear export of importin α. Furthermore, it has recently been shown that importin α is able to migrate into the nucleus in an importin β- and Ran-independent manner (Miyamoto *et al*, 2002). Although the issue of the physiological significance of the importin β-independent nuclear import of importin α is not yet resolved, it has very recently been reported that cellular stress, including UV irradiation, oxidative stress and heat shock, induces the nuclear accumulation of importin α, which results in the inhibition of conventional nuclear

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import (Miyamoto *et al*, 2004). However, the issue of whether importin α is able to carry cargoes into the nucleus in an importin β-independent manner in spite of the ability of importin α to translocate through the NPC by itself has not been resolved.

Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMKIV) is a multifunctional serine/threonine kinase and the phosphorylation of its substrates such as cAMP response element-binding protein (CREB) has been implicated in a number of cellular and physiological functions (for reviews, see Anderson and Kane, 1998; Krebs, 1998; Soderling, 1999). The phosphorylation of CREB at Ser^{133} by CaMKIV is crucial for the transcriptional activation of Ca^{2+}-induced CREB-mediated signaling pathways (Enslen *et al*, 1994; Matthews *et al*, 1994). CaMKIV is particularly important among the Ca^{2+}/calmodulin-dependent kinases because of its nuclear colocalization with CREB (Jensen *et al*, 1991; Matthews *et al*, 1994; Gao *et al*, 2004). The subcellular distribution of CaMKIV was observed to be rather dynamic; nuclear CaMKIV levels are increased in the neurons of the neocortex after the disruption of sensory input (Lalonde *et al*, 2004), whereas in granulosa cells of the ovary, CaMKIV migrates to the cytoplasm after a hormonal stimulus (Wu *et al*, 2000). Despite its importance in the nuclear localization and possible functional regulation by the dynamic movement within the cells, the nucleocytoplasmic transport mechanism of CaMKIV has not been elucidated.

In this study, using a digitonin-permeabilized cell-free transport assay (Adam *et al*, 1990), we demonstrate that the addition of importin α alone reproduced the nuclear import of CaMKIV and that the nuclear migration of CaMKIV required neither importin β nor Ran. The cytoplasmic microinjection of importin α-ΔIBB was found to facilitate the nuclear translocation of CaMKIV *in vivo*. Thus, our data show that CaMKIV is transported to the nucleus by importin α without the need for importin β. This is the first example to demonstrate that importin α can act as a nuclear import carrier like importin β family molecule. In addition, the possibility that the solitary nuclear migration of importin α and the importin α-driven nuclear import of CaMKIV penetrate the NPC in similar manners is demonstrated.

Results

CaMKIV is transported to the nucleus by a facilitated mechanism

The predicted molecular mass of CaMKIV is 53 kDa, suggesting that it could passively diffuse through the NPC. To determine empirically if CaMKIV migrates into the nucleus by diffusion or facilitated transport, *in vivo* microinjection assays with mammalian cell-expressed recombinant Flag-tagged CaMKIV were carried out. It has been established that wheat germ agglutinin (WGA) blocks facilitated transport, but not the free diffusion, of macromolecules through the NPC. To determine whether the nuclear translocation of CaMKIV is inhibited by WGA, CaMKIV and WGA were coinjected into the cytoplasm of NIH3T3 cells and the cells were incubated at 37°C for a period of up to 90 min. The nuclear migration of CaMKIV was inhibited by WGA, even after 90 min of incubation (Figure 1E), whereas the majority of the CaMKIV was translocated to the nucleus in the absence of WGA after 90 min of incubation (Figure 1D). These results

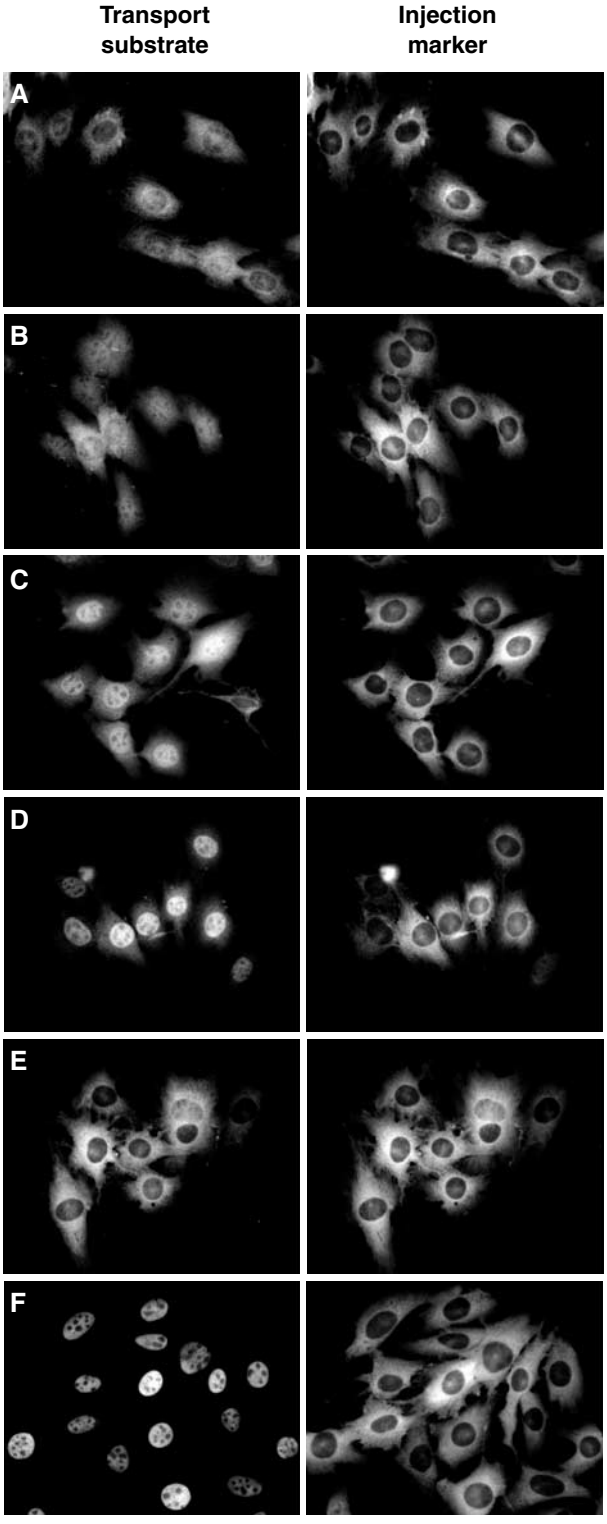

Figure 1 The slow and facilitated nuclear migration of CaMKIV. Flag-CaMKIV and GST-cNLS-CFP (10 μM each, left panels), along with GST-Venus (10 μM, right panels) as an injection marker, were cytoplasmically microinjected into NIH3T3 cells with or without WGA (2 mg/ml). The majority of Flag-CaMKIV remained in the cytoplasm during the first 15 min of incubation (**A**) and Flag-CaMKIV was gradually translocated to the nucleus with 30 (**B**), 60 (**C**) and 90 (**D**) min of incubation, whereas GST-cNLS-CFP was exclusively localized in the nucleus within 15 min after the injection (**F**). Coinjection of Flag-CaMKIV with WGA disrupted the transport of Flag-CaMKIV across the nuclear membrane even at 90 min after the injection (**E**).

indicate that CaMKIV is transported into the nucleus by facilitated transport, and not by passive diffusion.

Although the subcellular localization of endogenous or exogenously injected CaMKIV was predominantly nuclear, a cytoplasmic signal was also apparent in both cases. We observed that, when microinjected into the nucleus, CaMKIV was exported to the cytoplasm (data not shown), which explains the fact that CaMKIV is distributed throughout the cell. Despite functioning as a shuttling molecule between the nucleus and cytoplasm, it is suspected that the rate of nuclear translocation of CaMKIV is considerably slower than that of the classical NLS (cNLS). In fact, the migration of GST-cNLS-GFP to the nucleus was completed within 15 min (Figure 1F), whereas the majority of CaMKIV remained in the cytoplasm after 15 min incubation (Figure 1A). Approximately 60 min was required for CaMKIV to reach its predominantly nuclear localization (Figure 1B and C). Thus, the nuclear translocation of CaMKIV occurs by facilitated transport, but the underlying mechanism of the transport might be different from that of the classical transport system.

Importin α alone carries CaMKIV to the nucleus

Using digitonin-permeabilized semi-intact cells with mammalian cell-expressed Flag-CaMKIV and baculo-virally expressed GFP-CaMKIV (Figure 2J), we attempted to determine the factors required for the nuclear transport of CaMKIV. A cytosolic extract prepared from Ehrlich ascites tumor cells was found to contain the necessary components for transport, as described previously (Imamoto *et al*, 1995); both Flag-CaMKIV (Figure 2A) and GFP-CaMKIV (Figure 2G) were efficiently transported to the nucleus when the extract and an ATP regeneration system were added, while the nuclear transport of Flag-CaMKIV (Figure 2B) and GFP-CaMKIV (Figure 2H) was dramatically diminished when the cytosolic extract was omitted, indicating that the nuclear import of CaMKIV requires soluble transport factor(s).

In order to determine if CaMKIV is transported by classical transport factors, bacterially expressed importin α, importin β and Ran were used in semipermeable cells along with mammalian and insect cell-expressed CaMKIV and an ATP regeneration system. To our surprise, importin α alone had the highest activity for transporting CaMKIV to the nucleus without the addition of importin β or Ran (Figure 2C). In fact, the addition of importin β appeared to decrease the efficiency of transport, in both the presence and the absence of Ran (Figure 2D and E). To exclude the possibility that a residual amount of endogenous importin β in the permeabilized cells contributed to the nuclear migration of CaMKIV, the importin

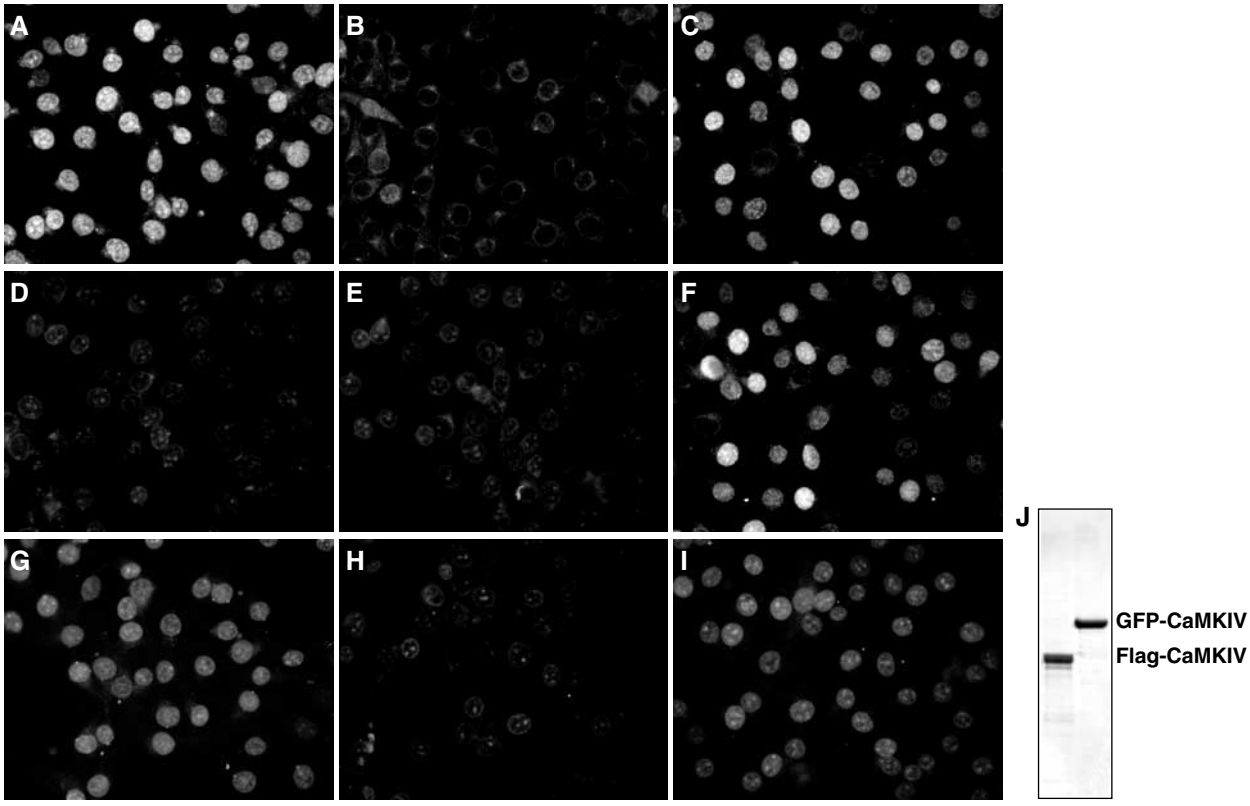

Figure 2 Importin α mediates the nuclear transport of CaMKIV without utilizing importin β or Ran. In digitonin-permeabilized BHK cells, FLAG-CaMKIV-His (3 μM) was incubated at 30°C for 40 min along with various transport factors. After fixation with 4% paraformaldehyde, the cells were immunostained with anti-CaMKIV antibody. Along with an ATP regeneration system, CaMKIV was transported to the nucleus when an Ehrlich ascites tumor cell extract (14 mg/ml) was added (**A**) but not when the extract was omitted (**B**). GST-importin α (24 μM) solely transported CaMKIV to the nucleus (**C**). Despite the addition of importin α, importin β (6 μM) reduced the efficiency of the transport in the presence (**D**) or absence (**E**) of Ran (8 μM). ΔIBB mutant of importin α (24 μM) carried CaMKIV into the nucleus (**F**). GFP-CaMKIV-His was similarly transported to the nucleus when Ehrlich ascites tumor cell extract (**G**) or GST-importin α-ΔIBB (**I**) was added, but not when soluble proteinaceous factors were omitted (**H**). Mammalian cell-expressed Flag-CaMKIV-His and baculo-virally expressed GFP-CaMKIV-His used in this study were shown by Coomassie staining (**J**).

β binding-deficient mutant (ΔIBB) of importin α was used instead of the full-length importin α. The addition of only importin α-ΔIBB indeed reproduced the apparent nuclear import of Flag-CaMKIV (Figure 2F) and GFP-CaMKIV (Figure 2I) with the same efficiency as the full-length importin α. These results strongly suggest that CaMKIV is transported to the nucleus by importin α alone without the need for importin β or Ran.

CaMKIV directly binds to importin α

Classical transport requires the formation of a ternary complex of importin α, importin β and cNLS-cargo in the cytoplasm. To determine whether or not CaMKIV directly interacts with importin α, mammalian cell-expressed Flag-CaMKIV was incubated with bacterially expressed GST-importin α or mutants thereof that were immobilized on glutathione-Sepharose. CaMKIV directly interacted with immobilized importin α (Figure 3A, lane 2), consistent with a recent report (Lemrow *et al*, 2004). CaMKIV also bound to importin α-ΔIBB (Figure 3A, lane 3), in agreement with the above experiment in which importin α-ΔIBB was shown to transport CaMKIV to the nucleus.

It is generally known that the binding of importin β to the IBB domain of importin α enhances the interaction between importin α and the cNLS. The reason for this is because the IBB domain has autoinhibitory activity and the binding of importin β to the IBB domain eliminates this autoinhibitory effect, thus creating easier access for cNLS-bearing proteins (Moroianu *et al*, 1996; Kobe, 1999; Fanara *et al*, 2000). In contrast, in digitonin-permeabilized cells, the nuclear transport of CaMKIV was found to be somewhat decreased when importin β was added (Figure 2D). To elucidate the reason for why importin β is not only unnecessary, but also has an inhibitory effect on the import of CaMKIV, we examined the *in vitro* binding between CaMKIV and transport factors in more detail. Immobilized GST-importin α was incubated with CaMKIV, along with increasing amounts of importin β. An excess amount of importin β disrupted the interaction between importin α and CaMKIV (Figure 3A, lanes 4–6), but not between importin α-ΔIBB and CaMKIV (Figure 3A, lane 7), indicating that the binding of importin β to the IBB domain suppresses the binding of CaMKIV to importin α. Moreover, no CaMKIV-bound importin β signal was found on a Western blot assay (Figure 3B, lane 4), indicating that CaMKIV does not bind to importin β directly. In addition, even in the presence of importin α, CaMKIV was unable to form a ternary complex with importin α and β (Figure 3B, lane 3), whereas immobilized GST-cNLS-GFP efficiently formed a ternary transport complex with importin α and β (Figure 3B, lane 1). The result with a crude cytosolic extract of Ehrlich ascites carcinoma cells confirmed that Flag-CaMKIV interacted with endogenous importin α, but not with endogenous importin β (Figure 3C, lane 3), whereas GST-cNLS-GFP formed a ternary complex with endogenous importin α and β (Figure 3C, lane 1). Moreover, other importin β family molecules such as transportin or importin 4 did not bind to the importin α/CaMKIV complex. These results are in good agreement with the result of the digitonin-permeabilized transport assay, which showed that CaMKIV was transported by importin α alone, and the transport was inhibited somewhat by the presence of importin β.

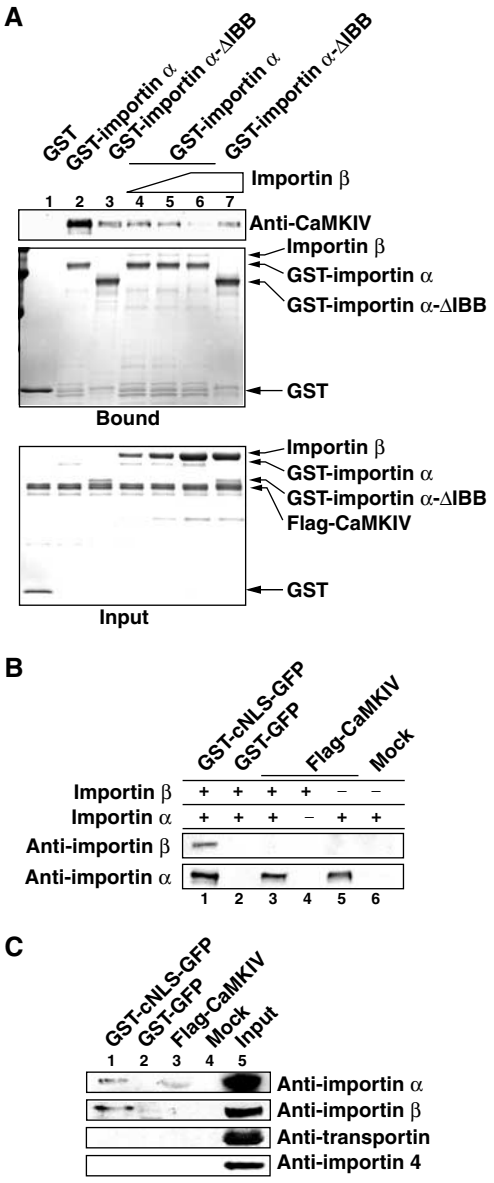

Figure 3 Interaction between importin α and CaMKIV. (**A**) GST-importin α (GST-imp α) and GST-importin α-ΔIBB (GST-imp α-ΔIBB) (15 pmol each) were immobilized on glutathione-Sepharose, and FLAG-CaMKIV (50 pmol) was applied to observe the interaction between importin α and CaMKIV. FLAG-CaMKIV interacted with both GST-importin α (lane 2) and GST-importin α-ΔIBB (lane 3), but not with GST alone (lane 1). The interaction between CaMKIV and importin α was disrupted by the addition of importin β (imp β). The amount of importin α-bound CaMKIV decreased as the amount of added importin β was increased from 100 pmol (lane 4) to 400 pmol (lane 6). Importin α-ΔIBB-bound CaMKIV was not affected by the addition of 400 pmol of importin β (lane 7). (**B**) Immobilized Flag-CaMKIV was used to examine the formation of a ternary complex with importin α and β. Importin α and/or β (100 pmol each) were added to immobilized Flag-CaMKIV (200 pmol) or GST-cNLS-GFP (100 pmol). A ternary complex of importin α, importin β and GST-cNLS-GFP was formed (lane 1). Importin β did not bind to importin α/CaMKIV complex (lane 3), nor to CaMKIV directly (lane 4). (**C**) Immobilized Flag-CaMKIV binds to importin α, but not to importin β, in a crude cytoplasmic extract of Ehrlich ascites tumor cells. The cytosolic extract was added to immobilized Flag-CaMKIV (6 nmol) or GST-cNLS-GFP (6 nmol). Importin β bound to a GST-cNLS-GFP/importin α complex (lane 1), but not to a CaMKIV/importin α complex (lane 3). Other importin β family molecules such as transportin or importin 4 did not bind to the CaMKIV/importin α complex (lower panels).

ATP is required for the nuclear translocation of CaMKIV but not as an energy source

The nuclear transport of CaMKIV was completely inhibited without the addition of exogenous ATP in digitonin-permeabilized cells, even in the presence of cytosolic extract (Figure 4A) or importin α (Figure 4B). Although at present, how ATP is used for the nuclear transport is a controversial issue, one interpretation is that ATP is required for the *de novo* synthesis of cellular GTP, which, in turn, is required for the maintenance of RanGTP/GDP gradient across the nuclear membrane (Schwoebel *et al*, 2002). However, when we substituted ATP with ATPγS, a nonhydrolyzable analog of ATP, importin α-driven transport of CaMKIV continued to be active in digitonin-permeabilized cells (Figure 4C) and Ran was not required for the nuclear import of CaMKIV *in vitro*. These results and the report showing that a non-ATP-binding mutant of CaMKIV is nuclear translocation-incompetent (Lemrow *et al*, 2004) raise the possibility that the binding of ATP to CaMKIV is required for its nuclear translocation rather than its use as an energy source. It is unlikely, however, that ATP is a prerequisite for the interaction between CaMKIV and importin α, since it was shown that the non-ATP-binding mutant of CaMKIV retains the ability to bind importin α (Lemrow *et al*, 2004). Thus, the issue of how ATP is used for the nuclear translocation of CaMKIV is yet to be determined.

Importin α-driven nuclear translocation of CaMKIV may utilize the same pathway as importin β-independent nuclear import of importin α

Importin α has been reported to migrate to the nucleus without utilizing importin β and Ran (Miyamoto *et al*, 2002). This report also concluded that the presence of an excess amount of the cNLS peptide dramatically inhibited the nuclear import of importin α in the absence of importin β and Ran. In order to further examine the possibility that both the unassisted migration of importin α to the nucleus and the importin α-driven nuclear translocation of CaMKIV use the

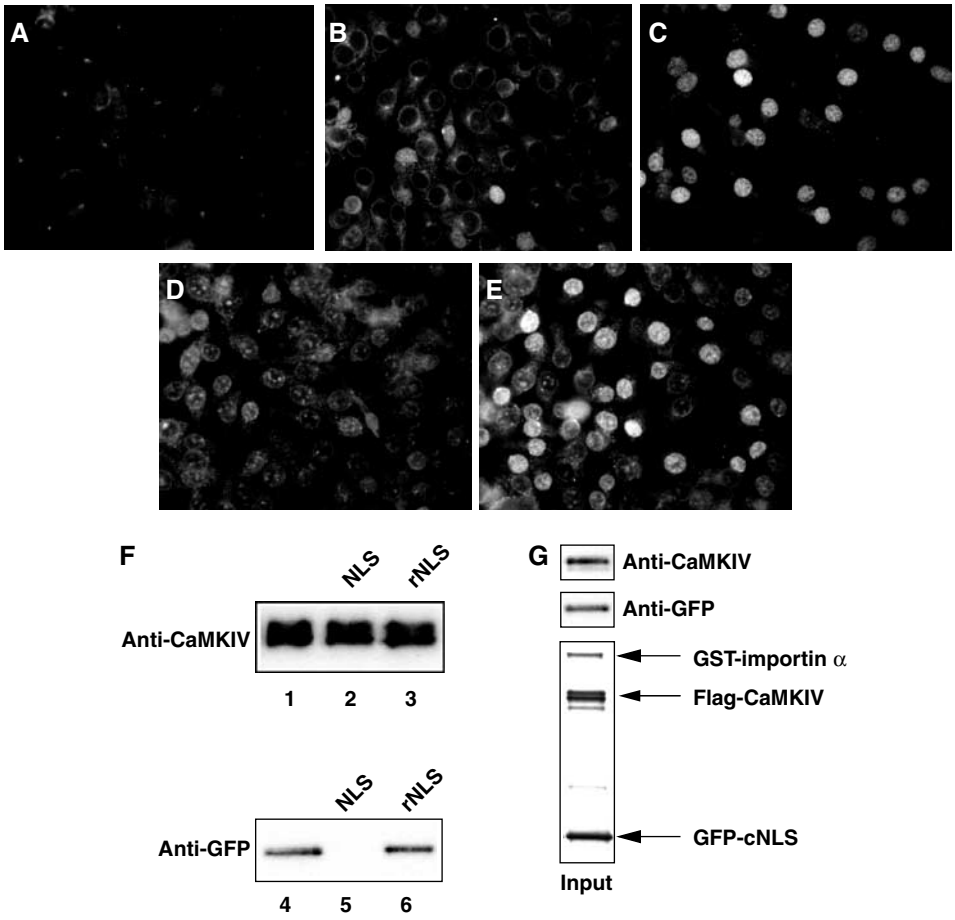

Figure 4 CaMKIV is transported to the nucleus in an ATP-dependent manner. In digitonin-permeabilized BHK cells, the nuclear transport of CaMKIV by cytosolic extract was inhibited when no ATP was added (**A**). Importin α-driven nuclear import of CaMKIV was also inhibited without ATP (**B**). The importin α-driven nuclear import of CaMKIV was restored when nonhydrolyzable ATPγS (3 mM) was added instead of ATP (**C**). In digitonin-permeabilized BHK cells, cNLS peptide or rNLS peptide (125 μM each) was added along with GST-importin α, FLAG-CaMKIV and an ATP regeneration system. The nuclear translocation of CaMKIV was inhibited by the addition of cNLS peptide (**D**), whereas the addition of rNLS did not affect the nuclear transport of CaMKIV (**E**). (**F**) cNLS does not disrupt the interaction between CaMKIV and importin α. GST-importin α (15 pmol) was immobilized on glutathione-Sepharose, and FLAG-CaMKIV (50 pmol) (upper) or GFP-cNLS (50 pmol) (lower) was added along with an excess amount of cNLS or rNLS peptides (6 nmol each). The interaction between CaMKIV and importin α was not affected by the addition of either peptide. (**G**) CaMKIV and cNLS simultaneously bind to importin α in an excess amount of cNLS. Flag-CaMKIV (50 pmol) and GFP-cNLS (250 pmol) were added to immobilized GST-importin α (15 pmol). Importin α-bound Flag-CaMKIV and GFP-cNLS were detected by anti-CaMKIV and anti-GFP antibodies, respectively.

same pathway, a peptide corresponding to the cNLS was added to the digitonin-permeabilized cells along with importin α and CaMKIV. As expected, the addition of the cNLS peptide dramatically reduced the nuclear import of CaMKIV (Figure 4D), while reversed NLS (rNLS) did not (Figure 4E).

We then attempted to determine if the inhibition was due to a blockage of the nuclear translocation of importin α rather than the disruption of the interaction between CaMKIV and importin α. Immobilized GST-importin α and GFP-cNLS were incubated with CaMKIV in the presence of an excess of the cNLS or rNLS peptide. Although GFP-cNLS was completely dissociated from importin α in an excess of cNLS peptide (Figure 4F, lower panel), the interaction between CaMKIV and importin α was not affected by cNLS peptide (Figure 4F, upper panel). In order to understand if cNLS and CaMKIV bind to importin α alongside, excess amounts of GFP-cNLS and Flag-CaMKIV were incubated with immobilized GST-importin α. As expected, both Flag-CaMKIV and GFP-cNLS were detected in the bound fraction of importin α (Figure 4G). These results indicate that both the importin β-independent nuclear translocation of importin α and importin α-driven nuclear translocation of CaMKIV are inhibited by the binding of cNLS to importin α in a similar fashion, suggesting that they may enter the nucleus using the same pathway.

CaMKIV binds to the C-terminal region of importin α

In spite of the fact that the binding of cNLS to importin α abolishes its ability to migrate to the nucleus independent of importin β, why does CaMKIV-bound importin α enter the nucleus? A possible explanation would be that the binding site for CaMKIV differs from that of the cNLS so that the binding of CaMKIV does not affect the ability of importin α to enter the nucleus. To test this hypothesis, the CaMKIV-binding region of importin α was determined using a variety of GST-tagged importin α deletion mutants. The immobilized GST-importin α mutants were incubated with Flag-CaMKIV or GFP-cNLS in an attempt to determine which domain of importin α is required for the interaction (Figure 5A). GST-importin α (1–459) showed efficient binding activity to CaMKIV (Figure 5B, lane 2), but the binding was dramatically reduced when GST-importin α (1–412) was used (Figure 5B, lane 3). GST-importin α (338–538) showed significant binding activity to CaMKIV (Figure 5B, lane 4), but a lower binding activity was observed for GST-importin α (425–538) (Figure 5B, lane 5). These data indicate that CaMKIV interacts with the C-terminal region of importin α, including armadillo repeats 9 and 10, which is clearly different from the binding site for cNLS. The result, at least in part, may explain the reason for why the importin β-independent nuclear migration of importin α is inhibited by cNLS, but not CaMKIV, although the issue of how the difference of binding sites affects the molecular state of importin α remains unknown.

The in vivo nuclear transport rate of CaMKIV is enhanced by the cytoplasmic microinjection of importin α-ΔIBB

In order to confirm the importin α-driven nuclear transport of CaMKIV *in vivo*, we attempted to inhibit the nuclear import of CaMKIV by microinjecting concentrated anti-importin α antibodies or C-terminal region-containing mutants of importin α, which might function dominant-negatively, but none of them succeeded (data not shown). This is probably due to the

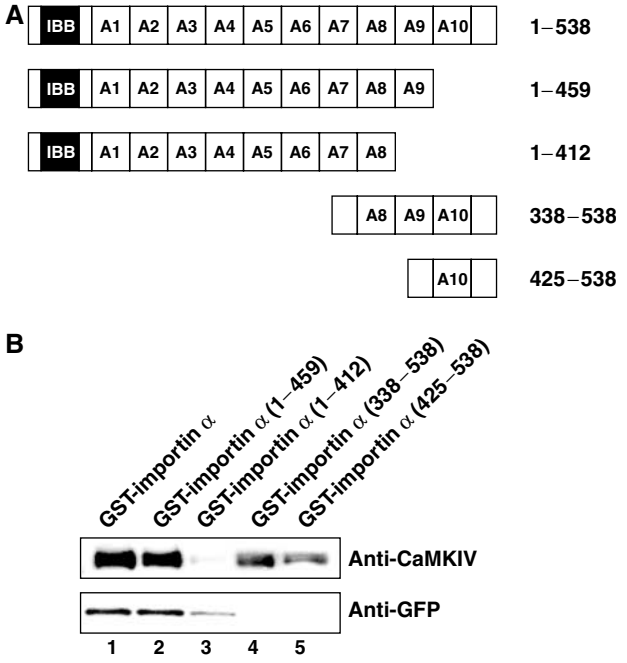

Figure 5 CaMKIV interacts with the C-terminal region of importin α. (**A**) Schematic representation of importin α and its mutants. (**B**) Flag-CaMKIV and GFP-cNLS were incubated with GST-importin α and its mutants immobilized on glutathione-Sepharose beads. GST-importin α (lane 1) and GST-importin α (1–459) (lane 2) showed binding activity to CaMKIV, whereas GST-importin α (1–412) (lane 3) showed dramatically reduced binding. GST-importin α (338–538) (lane 4) interacted with CaMKIV, and a somewhat reduced binding was observed with importin α (425–538) (lane 5). GFP-cNLS bound to all importin α mutants except the C-terminal deleted mutants (338–538) and (425–538) (lanes 4 and 5, lower).

diversity and abundance of importin α family molecules in intact cells. Therefore, we attempted to determine whether the nuclear transport rate of CaMKIV could be accelerated by the cytoplasmic microinjection of recombinant importin α-ΔIBB. The IBB region of importin α has been shown to be crucial for the nuclear transport of cNLS-bearing proteins (Görlich *et al*, 1996). It was therefore confirmed, as reported previously (Sekimoto *et al*, 1997), that the cytoplasmic microinjection of GST-cNLS-GFP along with GST-importin α-ΔIBB reduced the nuclear import rate of GST-cNLS-GFP (Figure 6C), but not with GST (Figure 6D). In contrast, 30 min after the coinjection of Flag-CaMKIV and GST-importin α-ΔIBB, the nuclear accumulation of CaMKIV was evident (Figure 6A), whereas the majority of CaMKIV remained in the cytoplasm in the case of the coinjection of Flag-CaMKIV with GST (Figure 6B). The opposite effects of importin α-ΔIBB on CaMKIV and cNLS support the notion that CaMKIV is transported to the nucleus by importin α without the need for importin β *in vivo*.

We then attempted to quantify the kinetics of the nuclear transport of CaMKIV with or without importin α-ΔIBB. Baculo-virally expressed GFP-CaMKIV was microinjected into the cytoplasm with GST-importin α-ΔIBB or GST, and the mean intensity of the nuclear fluorescence was measured at 3 min intervals. As shown in Figure 6E and F, the nuclear fluorescence of GFP-CaMKIV reached equilibrium faster as the result of coinjection with GST-importin α-ΔIBB. The time required to reach 50% of the maximum nuclear fluorescence

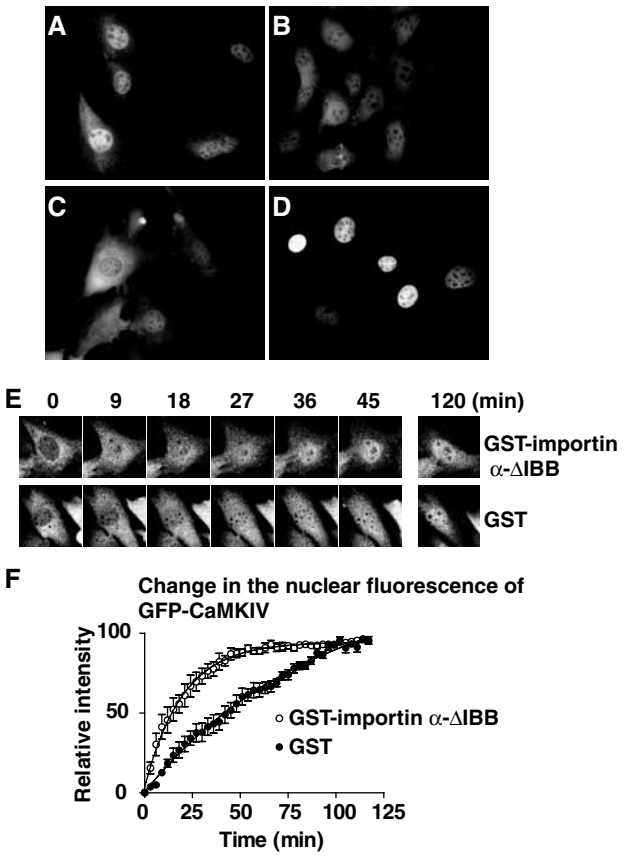

Figure 6 The nuclear transport rate of CaMKIV is enhanced by the cytoplasmic microinjection of importin α-ΔIBB *in vivo*. Flag-CaMKIV was microinjected into the cytoplasm of NIH3T3 cells together with GST-importin α-ΔIBB or GST. Flag-CaMKIV, when coinjected with GST-importin α-ΔIBB, accumulated in the nuclei (30 min incubation) (**A**), while much of the Flag-CaMKIV signal remained in the cytoplasm when coinjected with GST (30 min incubation) (**B**). The nuclear import of GST-cNLS-GFP was inhibited when coinjected with GST-importin α-ΔIBB (15 min incubation) (**C**), but not with GST (15 min incubation) (**D**). The nuclear translocation of GFP-CaMKIV in live cells was observed after the cytoplasmic microinjection with GST-importin α-ΔIBB (**E**, upper row) or GST (E, lower row). The relative nuclear fluorescence of microinjected cells was plotted for coinjection with GST-importin α-ΔIBB (**F**, empty circles) and with GST (F, filled circles). The time required to reach 50% of the maximum nuclear fluorescence was calculated for GST-importin α-ΔIBB ($t_{1/2} = 14.2$ min) and GST ($t_{1/2} = 41.7$ min) coinjections.

($t_{1/2}$) was determined for GST-importin α-ΔIBB ($t_{1/2} = 14.21$ min) and GST ($t_{1/2} = 41.69$ min) coinjection experiments. Thus, the microinjection of importin α-ΔIBB actually enhanced the rate of nuclear import of CaMKIV rather than decreasing it. These *in vivo* findings are in good agreement with the *in vitro* results that importin α alone mediates the nuclear import of CaMKIV in an importin β-independent manner.

Discussion

CaMKIV was originally given the name CaM Kinase-Gr because of its abundance in granule cells of the cerebellar cortex when it was initially identified as a nuclear kinase (Ohmstede *et al*, 1989; Jensen *et al*, 1991). Since then, numerous efforts have been made to understand the cellular functions and physiological significance of this enzyme. In many cases, it has been shown that CaMKIV functions as a nuclear kinase whose principal substrate is CREB, a transcriptional focal point of numerous cellular signaling pathways (for reviews, see Shaywitz and Greenberg, 1999; Johannessen *et al*, 2004). However, the issue of how it is transported into the nucleus has been unclear in spite of studies conducted over the past decade.

In this study, using a digitonin-permeabilized cell-free *in vitro* assay and *in vivo* microinjection techniques, we showed that importin α was able to carry CaMKIV to the nucleus alone without the need for importin β, although it has been proposed that importin α acts as an adapter molecule between importin β and cNLS-bearing proteins. Importin α-ΔIBB was also able to carry CaMKIV into the nucleus, thus confirming the solitary transport of CaMKIV by importin α without the aid of importin β. Importin β does not appear to be involved in the transport of CaMKIV. Therefore, the question arises as to whether or not importin α is capable of functioning as a nuclear transporter (carrier), similar to importin β. At least four requirements must be satisfied for a molecule to be classified as a nuclear transporter: the ability to form a complex with a cargo protein in the cytoplasm, the ability to penetrate through the NPC on its own, the ability to release the cargo in the nucleus and return to the cytoplasm.

Consistent with a recent report (Lemrow *et al*, 2004), we confirmed the first requirement, that is, the formation of a complex with CaMKIV, and also observed that importin α interacts with CaMKIV through the C-terminal region of importin α. On the other hand, the region of CaMKIV that is responsible for interaction with importin α has not yet been determined; attempts to narrow down the responsible domain have been unsuccessful to date (data not shown). We assume that a higher order structure of CaMKIV should be important for recognition by importin α, and any attempt to shorten the catalytic domain of CaMKIV would disrupt the structural integrity of the enzyme.

The inability of importin β to bind to the importin α/CaMKIV complex was confirmed by *in vitro* pull-down assays. It is unlikely that the binding of importin β is sterically hindered by importin α-bound CaMKIV since importin β binds to the N-terminal IBB region of importin α, which is separated a considerable distance from the armadillo repeats by a flexible linker region (Cingolani *et al*, 2000; Conti and Kuriyan, 2000; for review, see Goldfarb *et al*, 2004). On the other hand, the IBB domain is not required for the binding of CaMKIV since importin α-ΔIBB also was found to interact with CaMKIV as efficiently as full-length importin α. Thus, it is also unlikely that CaMKIV directly deprives the IBB domain of access to importin β. Therefore, we speculate that the binding of CaMKIV to importin α results in steric strain in importin α, which leads to a masking or engulfment of the IBB region.

The requirement of ATP for the nuclear translocation of CaMKIV is puzzling, since Ran is not required for the transport of CaMKIV. As reported recently (Lemrow *et al*, 2004), catalytic activity is important for the nuclear transport of CaMKIV, which apparently requires ATP; however, catalytic activity is not required for the interaction between CaMKIV and importin α. This would suggest that in addition to importin α, other factor(s) might be required for its transport. However, in digitonin-permeabilized cells, the only soluble factor required for the import of CaMKIV was importin α.

These results lead us to speculate that the catalytic activity of CaMKIV is utilized for insoluble factor(s) in the cells, such as the components of the NPC.

The second requirement is the ability of importin α to penetrate through the NPC without importin β. It has already been demonstrated that importin α alone migrates into the nucleus without the need for importin β or Ran (Miyamoto *et al*, 2002). We showed in this study that a complex of importin α with CaMKIV was able to translocate through the NPC in digitonin-permeabilized cells without the need for other soluble proteinaceous factors. Moreover, like the nuclear translocation of importin α alone, the importin α-mediated import of CaMKIV was effectively inhibited by the addition of the cNLS peptide without dissociation of the importin α/CaMKIV complex. These results suggest that both the CaMKIV/importin α complex and importin α alone penetrate through the NPC by the same pathway. How then, does cNLS inhibit transport? The IBB domain is known to function as an autoinhibitory region, which comprises a pseudo-NLS (Moroianu *et al*, 1996; Kobe, 1999; Fanara *et al*, 2000). It has been reported that the cNLS has a higher affinity toward importin α than the pseudo-NLS, so that the binding of cNLS to importin α releases the autoinhibitory effect of the IBB region. It is possible that the IBB region is an obstacle to the importin β-independent nuclear migration of importin α, and that to solely migrate to the nucleus, importin α must tuck the IBB domain. The concealing of IBB domain might be important for importin α to penetrate the NPC in an importin β-independent manner, and the inability of importin β to bind to the importin α/CaMKIV complex might be a resulting consequence of this.

CAS/RanGTP may cause the dissociation of the importin α/CaMKIV complex in the nucleus, which would be the third requirement, since both CAS and CaMKIV bind to the C-terminal region of importin α. However, the interaction between importin α and CaMKIV was not disrupted by the addition of CAS/Q69LRanGTP in the solution-binding assay (data not shown). On the other hand, in digitonin-permeabilized cells, we observed that the CaMKIV signal on the nuclear membrane disappeared on addition of CAS and Q69LRanGTP (data not shown). Therefore, as in yeast cells where Nup2p has been proposed to function as a nuclear release factor for cargo proteins from importin α (Gilchrist *et al*, 2002), mammalian nucleoporins on the nuclear side of the NPC might be required for the release. Alternatively, considering the relatively weak interaction between CaMKIV and importin α, nuclear release factors might not be essential but CaMKIV might be released from importin α on encountering its target molecules in the nucleoplasm.

The last requirement has already been extensively examined, which is CAS that exports importin α back to the cytoplasm (Kutay *et al*, 1997). Together with RanGTP, CAS forms a stable ternary complex with importin α, which is transported to the cytoplasm. RanGAP1 activates Ran to hydrolyze Ran-bound GTP, which causes the dissociation of the complex in the cytoplasm. The efficient release of the cargo in the nucleus and the export of importin α are sequential events, which might be functionally related. In digitonin-permeabilized cells, the amount of importin α required for the efficient entry of CaMKIV to the nucleus was greater than that for cNLS-containing substrates. This might be due to a lack of or a leak of as yet unidentified release factor(s) from the nucleus of permeabilized cells, so that the subsequent recycling of importin α may be reduced, which might, in turn, prevent the efficient import of CaMKIV *in vitro*.

From these results and the ensuring discussion, it is reasonable to conclude that importin α satisfies the requirements for a nuclear transporter. Therefore, we propose that importin α can function as a self-sufficient nuclear transporter of CaMKIV. The finding that importin α by itself is capable of importing at least one type of cargo protein allows us to consider the multiplicity and divergence of translocation steps through the NPC, and it would be interesting to know if CaMKIV is simply an exception to the classical transport system, or that a number of cargo proteins are transported by importin α without utilizing importin β.

Materials and methods

Cell culture

NIH3T3 and BHK cells were cultured in DMEM (Sigma) supplemented with heat-inactivated 10% fetal bovine serum (EQUITECH-BIO) at 37°C in 10% CO_2.

Construction of mammalian expression vectors

pCS2-Flag-CaMKIV-His was constructed by inserting a PCR fragment of mouse CaMKIV to *Xho*I sites; the fragment was amplified by Flag- and His-attached primers. pEGFP-C1-CaMKIV was constructed by subcloning the CaMKIV fragment to *Xho*I and *Sac*II sites of pEGFP-C1 vector (Clontech). All coding regions of the vectors were DNA-sequenced to confirm the correct reading frames.

Cell-free import assay

Digitonin-permeabilized BHK cells were prepared essentially as described previously (Adam *et al*, 1990), with the following modifications. Permeabilized cells were incubated at 4°C for 10 min and washed twice with transport buffer (20 mM HEPES–KOH pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 1 mM DTT, 1 μg each of aprotinin, pepstatin and leupeptin) to minimize residual proteins in the cytoplasm. The cells were incubated at 30°C for 40 min with 2% BSA, transport substrates/factors and an ATP regeneration system in a total volume of 10 μl per sample. After the incubation, the cells were fixed with 4% paraformaldehyde in transport buffer, followed by permeabilization of the nuclear membrane by 0.05% Triton X-100 in PBS and immunostaining of CaMKIV by a primary anti-CaMKIV monoclonal antibody (Transduction Laboratories) and a secondary Alexa546 anti-mouse IgG antibody (Molecular Probes).

Transfection assays

The transfection of NIH3T3 cells was carried out using Effectene (Qiagen) according to the manufacturer's operation manual. NIH3T3 cells were grown on a 35 mm Glass Bottom Culture Dish (MatTek). For each expression vector, 400 ng of plasmid was transfected per dish, and the cells were incubated for 16 h before observation under a Zeiss Axiovert 200M microscope with Plan-Neofluar ×40/0.75 Ph2 objective lens.

Preparation of recombinant proteins, cell extract and peptides

The expression of FLAG-CaMKIV-His in mammalian cells was performed using Freestyle (Invitrogen) according to the manufacturer's operation manual. Briefly, 100 μg of pCS2-FLAG-CaMKIV-His and 100 μl of 293fectin (Invitrogen) were separately mixed with 3 ml of Opti-MEM (Invitrogen). After an appropriate period of incubation, the lipofection mixture was transferred to 293F cells (1.0×10^8 cells in 100 ml Expression Medium (Invitrogen)) in a 250 ml Erlenmeyer flask with a gas-permeable cap. The cells were incubated for 48 h in an 8% CO_2 atmosphere at 37°C on a rotary shaker revolving at 150 r.p.m. The cells were collected by centrifugation and lightly sonicated in lysis buffer (20 mM Tris–HCl pH 8.5, 500 mM KCl, 5 mM β-mercaptoethanol, 10 mM imidazole, 1 μg each of aprotinin, pepstatin and leupeptin). After ultracentrifugation, the supernatant was subjected to one-step

purification of FLAG-CaMKIV-His using Ni-NTA (Qiagen) according to the manufacturer's instructions. The buffer was changed to transport buffer before freezing. All other bacterially expressed recombinant proteins were purified as reported previously (Sekimoto *et al*, 1997). Ehrlich ascites cell extract was prepared essentially as described previously (Imamoto *et al*, 1995), with the modification that the concentration of the extract was carried out using an ultrafiltration device (Vivascience). Peptides for cNLS (CYGGPKKKRKVEDP) and reverse NLS (CYGGPDEVKRKKKP) were prepared in transport buffer.

In vitro binding between CaMKIV and importin α

GST-tagged recombinant importin α (wild type and its mutants, 15 pmol each) was immobilized on glutathione-Sepharose (Pharmacia). Recombinant FLAG-CaMKIV-His (50 pmol) and the following competitors were added to the samples: 6 nmol NLS peptide, 6 nmol reverse NLS peptide, 300 pmol Q69LRanGTP, 200 pmol CAS and 400 pmol importin β. The samples were incubated on a rotator for 5 h at 4°C. The glutathione-Sepharose beads were washed twice with washing buffer (20 mM Tris–HCl pH 8.5, 500 mM KCl, 1 mM DTT, 1 μg each of aprotinin, pepstatin and leupeptin) and twice with transport buffer. SDS–PAGE was carried out on a 4–20% gradient acryl amide gel (Daiich Pure Chemicals). After blotting to a nitrocellulose membrane, the bands were visualized by a primary antibody (anti-CaMKIV, Transduction Laboratories) and a secondary antibody (HRP-conjugated anti-mouse IgG, Pierce). Chemiluminescence was detected using HRP substrate (Pierce) and luminescence photography system (Atto).

Microinjection and time-lapse imaging

NIH3T3 cells on a 35 mm Glass Bottom Culture Dish were cytoplasmically microinjected using a micromanipulator system (Narishige). Samples were prepared in transport buffer (20 mM HEPES–KOH pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 1 mM DTT, 1 μg each of aprotinin, pepstatin and leupeptin) and filtered through a 0.22 μm PVDF filter (Millipore) prior to the injection. The concentrations of the injected samples were as follows: 130 μM GST-importin α-ΔIBB, 130 μM GST, 10 μM Flag-CaMKIV-His, 10 μM GFP-CaMKIV, 10 μM GST-cNLS-CFP and 10 μM GST-Venus (Nagai *et al*, 2002) as an injection marker. For time-lapse imaging, the micromanipulator was assembled on a Zeiss LSM510Meta confocal system equipped with a heater and CO_2-supplying system to keep the cells at 37°C in 10% CO_2 during the observation. The recording of images started within 3 min after the first injection and was continued for a period of up to 2 h at 3 min intervals. The optical parameters were as follows: argon 488 nm laser for GFP excitation, Plan-Neofluar ×40/0.75 Ph2 objective lens, 133 μm pinhole and Z-stack of 2 μm interavals with a total of seven pictures for each time interval. The Z-sections were stacked together and the mean nuclear fluorescence was measured using bundled software (Zeiss). A total of five cells were statistically analyzed for each sample. The relative intensity in the nucleus was fitted for the function $R=M(1-e^{-kt})$, where R is the relative intensity in the nucleus, M is the maximum level of the nuclear intensity at equilibrium, k is the rate constant and t is time in minutes. The curves were fitted using Prism 4 (GraphPad).

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