Nuclear export of LIM-kinase 1, mediated by two leucine-rich nuclear-export signals within the PDZ domain

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LIM-kinase 1 (LIMK1) is a serine/threonine kinase that phosphorylates cofilin and regulates actin-filament dynamics. LIMK1, which contains two LIM domains and a single PDZ domain, localizes predominantly in the cytoplasm, but its mutant, deleted with the PDZ domain, localizes mainly in the nucleus, thereby indicating that the PDZ domain plays a role in the cytoplasmic localization of LIMK1. Here we provide evidence that the PDZ domain of LIMK1 contains two functional leucine-rich nuclear-export signals (NESs). The PDZ domain of LIMK1 fused with glutathione S-transferase (GST–PDZ), when injected into the nucleus, was rapidly excluded from the nucleus, but its mutant with replacements of conserved hydrophobic residues in two putative NESs by alanines remained in the nucleus. The nuclear export of GST–PDZ was sensitive to leptomycin B (LMB), a specific inhibitor of nuclear export mediated by leucine-rich NESs. Malfunctional mutation of two NESs or LMB treatment prevented the nuclear export of full-length LIMK1 and induced its nuclear accumulation. These results suggest that the predominant localization of LIMK1 in the cytoplasm is supported by two NESs within the PDZ domain and that LIMK1 normally shuttles between the cytoplasm and the nucleus. We also provide evidence that a short basic cluster sequence within the protein-kinase domain is involved in the nuclear import of LIMK1.

Key words: LIM-kinase, nuclear-export signal, nuclear-localization signal.

INTRODUCTION

LIM-kinase 1 (LIMK1) is a member of a novel subclass of protein serine/threonine kinases, found recently to be present in various vertebrates [1–9]. The LIMK family kinases, composed of LIMK1 and LIMK2, possess characteristic structural features, consisting of two LIM domains and a PDZ domain in the N-terminal half and a protein-kinase domain in the C-terminal half [1–9]. The LIM domain, consisting of two closely adjacent zinc fingers, is present in diverse proteins and is recognized as the motif involved in protein–protein interactions [10]. The PDZ domain, a 80–100 amino acid motif found singly or multiply in various sub-membranous and cytoplasmic proteins, is also known to function as the protein-binding motif [11]. The LIM and PDZ domains in LIMKs are thought to be involved in regulation of the kinase activity or in subcellular localization through protein–protein interactions. The kinase domains of LIMKs contain a consensus sequence of protein kinases, but are unique in that they have an unusual sequence motif in the kinase catalytic loop in subdomain VIB and a highly basic insert sequence between subdomains VII and VIII [1–9]. This basic cluster sequence was previously assumed to function as a nuclear-localization signal (NLS) [1], but experimental evidence has been awaited.

LIMK1 is expressed in various cultured cells, including fibroblasts, and epithelial and haematopoietic cell lines [1]. LIMK1 directly binds to actin filaments and when transfected into cultured cells it markedly induces actin cytoskeletal reorganization [12]. We and other groups recently provided evidence that LIMK1 phosphorylates cofilin at Ser-3 and mediates Rac-induced actin reorganization [12,13]. Cofilin is a potent regulator of actin-filament dynamics [14,15]. As the actin-binding and -depolymerization activity of cofilin is negatively regulated by phosphorylation at Ser-3 [16,17], LIMK1 seems to play a key role in regulating actin-filament dynamics through phosphorylating cofilin. LIMK1 is highly expressed in neurons in developing mammals [18]. Genetic studies implicated hemizygotic deletion of the LIMK1 gene as the cause of impairment of visuospatial cognition of Williams ' syndrome [19]. Thus, LIMK1 may play a role in the development or maintenance of neuronal circuitry that mediates cognitive functions.

LIMK1, when transfected in cultured cells, localized mainly in the cytoplasm [4], but the mutant with the PDZ domain deleted localized mainly in the nucleus [20]. When the normally nuclear cyclin A was fused with the PDZ domain of LIMK1, it localized in the cytoplasm [20]. These results suggest that the PDZ domain has a function to localize the protein in the cytoplasm. Furthermore, glutathione S-transferase (GST) fused with the PDZ domain of LIMK1 (GST–PDZ), when injected into the nucleus, was rapidly excluded from the nucleus, indicating that the PDZ domain of LIMK1 probably has nuclear-export activity [20]. The nuclear export of large macromolecules is an active-transport process mediated by nuclear-export signal (NES) sequences [21–23]. Three types of NES sequence have been identified [21–23]. Among them, a leucine-rich-type NES possessing characteristic spacing of leucine or other hydrophobic residues was characterized in various proteins, including Rev protein of HIV-1, an inhibitor of cAMP-dependent protein kinase (PKI-α), mitogen-activated protein kinase kinase, actin, zyxin and c-Abl [24–29]. Recent studies revealed that CRM1 (also called exportin 1) is the receptor for leucine-rich NES and that leptomycin B (LMB) specifically inhibits the nuclear export mediated by leucine-rich NES by blocking the interaction between NES and CRM1 [30–35].

Abbreviations used: GFP, green fluorescent protein; GST, glutathione S-transferase; LIM, acronym of Lin-11, Isl-1 and Mec-3; LIMK, LIM-kinase; LMB, leptomycin B; NES, nuclear-export signal; NLS, nuclear-localization signal; PDZ, acronym of PSD-95, Dlg and ZO-1; PKI-α, inhibitor of cAMP-dependent protein kinase.

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We present here evidence that the PDZ domain of LIMK1 contains two functional leucine-rich NESs and that the cytoplasmic localization of LIMK1 is supported by these NESs. Disruption of two NESs or treatment with LMB abrogated the nuclear-export activity of LIMK1 and induced the accumulation of full-length LIMK1 in the nucleus. This means that LIMK1 normally shuttles between the nucleus and the cytoplasm. We also provide evidence that an NLS-like short basic sequence within the protein-kinase domain is involved in the nuclear import of LIMK1.

EXPERIMENTAL

Plasmid construction

The primers used for PCR amplification were as follows: A, 5′-ATGGGCCCACCCCCGTCATCGAG-3′; B, 5′-ATGTCGACGCATGCACATTTCGGATGGGCGTGCC-3′; C, 5′-AAGCATGCCCGCAGACGAGGCAGACGCACTGATTCAGGAAACCAGCCGC-3′; D, 5′-GCACTAGTCTCCCCGTGGATGAGGTC-3′; E, 5′-ATGTCGACGCATGCGGCTGGTTTCCTGAATCAG-3′; F, 5′-AAGCATGCGCACTCCAGGCAACCCTCGAGCATGACCCTCAC-3′; G, 5′-ATGTCGACACCCCCGTCATCGAG-3′; H, 5′-ATGCGGCCGCTCAGCTCCTCAAGACAGGTTTCTG-3′; I, 5′-AGCTGACCCTCGAGCATGAC-3′; J, 5′-AGGAATTCCAGGCCCTCAGGCTGAGTCT-3′; K, 5′-ACGAATTCAAGCGCTACACCGTGGTGGG-3′; and L, 5′-GCTCTAGAGGCCTCAGGCCATAGTCGGGGACGTCATAGGGGGGTAAGGCAGTCCGCTCTCGCC-3′.

The cDNA fragments of the A3, A2 and A5 mutants of LIMK1 (see Figure 3A, below) were obtained as follows. For the A3 mutant, the cDNA for human LIMK1(144–234) was amplified by PCR using primers A and B, cut with *Apa*I and *Sal*I, and ligated into pBluescript II SK$^-$ (Stratagene) to yield pBS-LK1(144–234). Then the cDNA for A3 mutant of LIMK1(235–343) was amplified by PCR, using primers C and D, cut with *Sph*I and *Spe*I, and ligated into the pBS-LK1(144–234). The resulting plasmid was cut with *Sph*I and blunt-end ligated to yield pBS-LK1(144–343)(A3) coding for the A3 mutant of LIMK1(144–343). For the A2 mutant, the cDNA for LIMK1(144–248) was amplified by PCR using primers A and E, cut with *Apa*I and *Sal*I, and ligated into pBluescript II SK$^-$ to yield pBS-LK1(144–248). Next, the cDNA for the A2 mutant of LIMK1(249–343) was amplified by PCR using primers F and D, cut with *Sph*I and *Spe*I, and ligated into pBS-LK1(144–248). The resulting plasmid was cut with *Sph*I and blunt-end ligated to yield pBS-LK1(144–343)(A2) coding for the A2 mutant of LIMK1(144–343). For the A5 mutant, the cDNA coding for LIMK1(144–248)(A3) was amplified by PCR, using primers A and E, and pBS-LK1(144–343)(A3) as a template, cut with *Apa*I and *Sal*I, and ligated into pBluescript II SK$^-$ to yield pBS-LK1(144–248)(A3). The cDNA fragment of the A2 mutant of LIMK1(249–343) was then amplified as above, cut with *Sph*I and *Spe*I, and ligated into pBS-LK1(144–248)(A3). The resulting plasmid was cut with *Sph*I, and blunt-end ligated to yield pBS-LK1(144–343)(A5) coding for A5 mutant of LIMK1(144–343).

The plasmid for GST–PDZ, GST fused with LIMK1(144–296), was constructed in pGEX-4T-3 vector (Pharmacia Biotech) as described previously [20]. The plasmids for GST–PDZ mutants, A3, A2 and A5, were constructed by PCR amplification, using primers G and H and pBS-LK(144–343)(A3), -(A2) and -(A5) as the templates, respectively, followed by digestion with *Sal*I and *Not*I and ligation into pGEX-4T-3. The plasmid for C-terminally Flag-tagged LIMK1 was generated as described [12,36]. To obtain the expression plasmid for Flag-tagged LIMK1(A5), pBS-LK1(144–343)(A5) was cut with *Eco*RI and *Xho*I, and replaced with the original *Eco*RI–*Xho*I fragment of pBS-Flag-LIMK1(D460A) [12], and the resulting plasmid was digested with *Not*I and inserted into pRc-CMV vector (InVitrogen). The plasmid coding for GFP–LIMK1, green fluorescent protein (GFP) fused with LIMK1(215–647), or its mutant GFP–LIMK1(A5), was constructed by inserting the *Eco*RI–*Sac*II fragment of LIMK1 or LIMK1(A5) cDNA into the pEGFP-C1 vector (Clontech). The cDNA for GFP–LIMK1(A5ΔNLS), in which a basic NLS-like sequence (residues 496–504) of GFP–LIMK1(A5) is replaced by Glu-Phe, was constructed by ligation of cDNA fragments, amplified by PCR, using primers I and J, and K and L, at an *Eco*RI site. In this study, we used plasmids coding for the kinase-inactive form of LIMK1, LIMK1(D460A), in which Asp-460 is replaced by Ala, because with the kinase-active forms, it is difficult to visualize clearly the nucleocytoplasmic localization.

Purification of GST-fusion proteins

GST-fusion proteins were expressed in *Escherichia coli* and purified on glutathione–Sepharose 4B (Pharmacia), as described previously [37].

Cell cultures, transfection and microinjection

COS-7 and HeLa cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with the plasmid DNA by the lipofectamine (Gibco-BRL) method. For microinjection, COS-7 cells were grown on glass coverslips and cultured to around 70% confluency. Purified GST-fusion protein (2 mg/ml) was mixed with a reporter rabbit IgG (1 mg/ml) in PBS and injected into the nucleus of cultured cells, using a Micromanipulator-Transjector system (Zeiss-Eppendorf, Tokyo, Japan). After injection, the cells were incubated at 37 °C for 30 min and fixed.

Immunostaining

Cells were plated on 24-mm glass coverslips. At 36 h after transfection or 30 min after microinjection, cells were fixed and permeabilized, as described previously [20]. To visualize GST-fusion proteins, cells were stained with anti-GST monoclonal antibody (Santa Cruz Biotechnology), followed by FITC-labelled anti-mouse IgG antibody (Amersham). Rabbit IgG was visualized by Texas Red-labelled donkey anti-rabbit IgG antibody (Amersham). Flag-tagged LIMK1 was visualized using M2 anti-Flag monoclonal antibody (Kodak), followed by FITC-labelled anti-mouse IgG antibody (Amersham). Fluorescence was viewed and photographed using an epifluorescent microscope (Carl Zeiss, Tokyo, Japan).

RESULTS

Nuclear export of the PDZ domain of LIMK1 is inhibited by LMB

When GST–PDZ, a GST fusion protein with the PDZ domain of LIMK1, was injected into the nucleus of COS-7 cells, it was almost completely excluded from the nucleus within 30 min of injection, whereas rabbit IgG, co-injected as a marker of the injection sites, was retained in the nucleus (Figure 1, −LMB). To determine if the nuclear export of GST–PDZ is mediated by the leucine-rich NES previously characterized in Rev, PKI-α and other proteins [24–29], prior to microinjection we treated the cells with LMB, a specific inhibitor for leucine-rich NES-

dependent nuclear export. This pretreatment almost completely blocked the nuclear export of GST–PDZ, and it was retained in the nucleus (Figure 1, +LMB). Thus, the PDZ domain of LIMK1 probably contains the leucine-rich NES(s).

Nuclear export of the PDZ domain of LIMK1 is mediated by two tandemly aligned leucine-rich NESs

Comparison of the primary sequence of the PDZ domain of LIMK1 with previously characterized leucine-rich NESs revealed that it contains two tandemly aligned potential NES sequences, NES-1 (residues 231–242) and NES-2 (residues 243–255), which have characteristic spacing of hydrophobic residues (ΨxxΨxΨ, in which Ψ indicates hydrophobic residues; Figure 2). To determine whether these two sequence motifs actually function as NESs, we constructed three GST–PDZ mutants (A3, A2 and A5) in which three hydrophobic residues (Leu-236, Ile-239 and Leu-241) in NES-1 (A3), two leucines (Leu-249 and Leu-252) in NES-2 (A2) or all five residues (A5) were replaced by alanines to disrupt NES-1, NES-2, or both, respectively (Figure 3A). These GST–PDZ mutant proteins were purified (Figure 3B) and injected into the nucleus of COS-7 cells along with a reporter rabbit IgG (Figure 3C). GST–PDZ(A3) and GST–PDZ(A2) were rapidly excluded from the nucleus within 30 min of injection, with no obvious difference from wild-type GST–PDZ. In contrast, GST–PDZ(A5) remained in the nucleus. The nuclear export of GST–PDZ(A3) and GST–PDZ(A2) was inhibited by LMB. These findings suggest that the nuclear export of the PDZ domain of LIMK1 is the result of actions of these two NESs. As the mutation of one of two putative NESs did not inhibit the nuclear export of GST–PDZ, both NES-1 and NES-2 individually have the potential to export the protein from the nucleus.

In a previous study, we presented data that the B region (residues 196–247) within the PDZ domain of LIMK1, when fused with cyclin A, has activity to localize the normally nuclear protein cyclin A in the cytoplasm [20]. Since the B region contains the intact NES-1 sequence, cytoplasmic localization activity of the B region is probably related to the nuclear-export activity of NES-1. On the other hand, GST–PDZΔB (GST–PDZ with the B region deleted), when injected into the nucleus, was not exported from the nucleus [20], even though the leucine-rich core sequence of NES-2 (LLQLTL, residues 249–254, where underlining represents hydrophobic residues conserved in nuclear export signals) was present. Therefore, NES-2 probably requires the N-terminally extended sequence, in addition to the leucine-rich core, to exert nuclear-export activity.

Nuclear accumulation of LIMK1 by disruption of NESs or LMB treatment

As reported previously [4,20], when the full-length LIMK1 was expressed in HeLa cells, this kinase predominantly localized in the cytoplasm (Figure 4A). To determine if two NESs in the PDZ domain described above contribute to the cytoplasmic localization of LIMK1, two experiments were done. First, we transfected into HeLa cells a mutant of full-length LIMK1, LIMK1(A5), in which five hydrophobic residues (Leu-236, Ile-239, Leu-241, Leu-249 and Leu-252) in NES-1 and NES-2 were replaced by alanines to destroy the nuclear-export activity of both NESs; we then examined the nuclear-cytoplasmic localization in transfected cells by indirect immunofluorescence staining (Figure 4A). In contrast with the wild-type LIMK1, LIMK1(A5) localized mainly in the nucleus, indicating that these two NESs are indeed required for cytoplasmic localization of LIMK1 and that they function to export full-length LIMK1

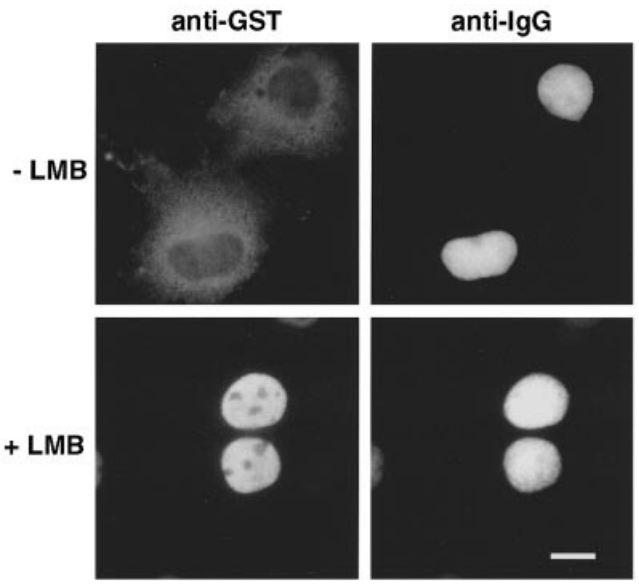

Figure 1 The nuclear export of GST–PDZ protein is sensitive to LMB

A mixture of GST–PDZ and a rabbit reporter IgG was microinjected into the nucleus of COS-7 cells. After incubation for 30 min at 37 °C, cells were fixed and doubly stained with anti-GST antibody followed by FITC-labelled second antibody (left panels) and Texas Red-labelled anti-rabbit IgG antibody (right panels). +LMB, cells pretreated with LMB (4 ng/ml) for 60 min at 37 °C, prior to microinjection. Scale bar, 20 μm.

from the nucleus. We next examined the effect of LMB on cytoplasmic localization of full-length LIMK1 expressed in HeLa cells. After treatment (1 h) with LMB, full-length LIMK1 was concentrated in the nucleus (Figure 4A, LIMK1+LMB). The LMB-induced nuclear concentration of LIMK1 was also observed in COS-7 cells transfected with LIMK1 (results not shown). To examine the time course of nuclear accumulation of LIMK1 during LMB treatment, GFP-fusion protein of LIMK1 (GFP–LIMK1) was expressed in COS-7 cells. GFP–LIMK1 localized in the cytoplasm prior to LMB treatment, but it began

LIMK1	NES-1	IRNVPLDEIDLL	(231-242)
LIMK1	NES-2	IQETSRLLQLTLE	(243-255)
HIV Rev	NES	LQLPPLERLTLD	(71-82)
PKI-α	NES	ELALKLAGLDIN	(36-47)
MAPKK	NES	ALQKKLEELELD	(32-43)
α-Actin	NES-1	ALPHAIMRLDLA	(170-181)
α-Actin	NES-2	DIKEKLCYVALD	(211-222)
c-Abl	NES	KLESNLRELQIC	(1056-1067)
Zyxin	NES	LTMKEVEELELL	(319-330)
Consensus		$\Psi X_n \Psi X_2 \Psi X \Psi$	

Figure 2 Alignment of potential NES sequences of LIMK1 with previously characterized leucine-rich NESs

NES sequences in HIV Rev, PKI-α, mitogen-activated protein kinase kinase (MAPKK), actin, c-Abl and zyxin are from [24–29]. Grey shading, three highly conserved hydrophobic residues. Consensus sequence is shown at the bottom. Ψ indicates hydrophobic residues, which include leucine, isoleucine and valine.

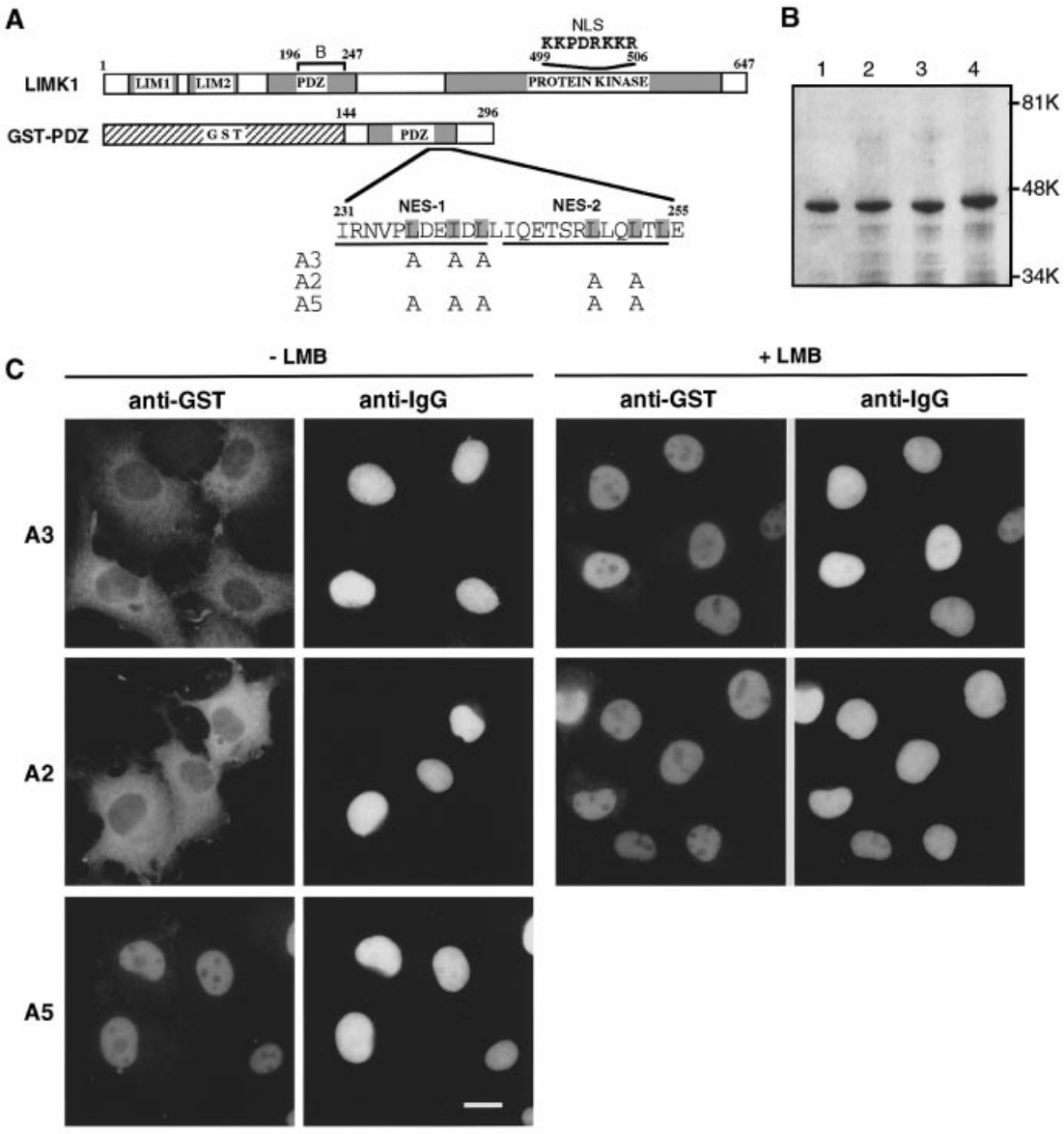

Figure 3 The nuclear export of GST–PDZ is mediated by two NESs, NES-1 and NES-2

(**A**) Structures of LIMK1, GST–PDZ and its mutants. In LIMK1, grey boxes indicate two LIM domains, a PDZ domain and a kinase domain. The numbers above the boxes indicate the amino acid residue numbers of human LIMK1 [1]. B indicates a region described in our previous paper [20]. Three GST–PDZ mutants (A3, A2 and A5) have replacements of hydrophobic residues in NES-1 and NES-2 by alanines, as indicated. (**B**) SDS/PAGE analyses of GST–PDZ fusion proteins. Proteins purified on glutathione–Sepharose were separated on SDS/PAGE and stained with Coomassie Brilliant Blue. Lane 1, GST–PDZ; lane 2, GST–PDZ(A3); lane 3, GST–PDZ(A2); lane 4, GST–PDZ(A5). The positions of size-marker proteins are indicated on the right. (**C**) A mixture of GST–PDZ mutant and a rabbit reporter IgG was injected into the nucleus of COS-7 cells. After incubation for 30 min at 37 °C, cells were fixed and doubly stained with anti-GST and anti-rabbit IgG antibodies, as described in Figure 1. +LMB, cells pretreated with LMB (4 ng/ml) for 60 min at 37 °C before microinjection. Scale bar, 20 μm.

to appear in the nucleus within 15 min of LMB treatment and was highly concentrated in the nucleus within 60 min (Figure 4B). Taken together, these observations suggest that the predominant localization of LIMK1 in the cytoplasm is supported by two leucine-rich NESs within the PDZ domain. Furthermore, the finding that LIMK1 accumulated in the nucleus by disruption of NESs or LMB treatment suggests that LIMK1 normally shuttles between the cytoplasm and the nucleus, rather than being stably localized in the cytoplasm. As the molecular mass of LIMK1 ($\approx$ 73000 Da) is higher than the masses ($<$ 40000–60000 Da) predicted for proteins freely diffusible between the cytoplasm and the nucleus, it is probable that LIMK1 is imported into the nucleus by active-transport mechanisms.

Deletion of a short basic cluster sequence within the protein-kinase domain represses nuclear accumulation of LIMK1(A5)

As LIMK1 contains an NLS-like basic cluster sequence (residues 499–506, KKPDRKKR) within the kinase domain, we next asked if this sequence is responsible for the nuclear import of LIMK1. We constructed two GFP–LIMK1 mutants: GFP–LIMK1(A5), in which two NESs were disrupted, and GFP–

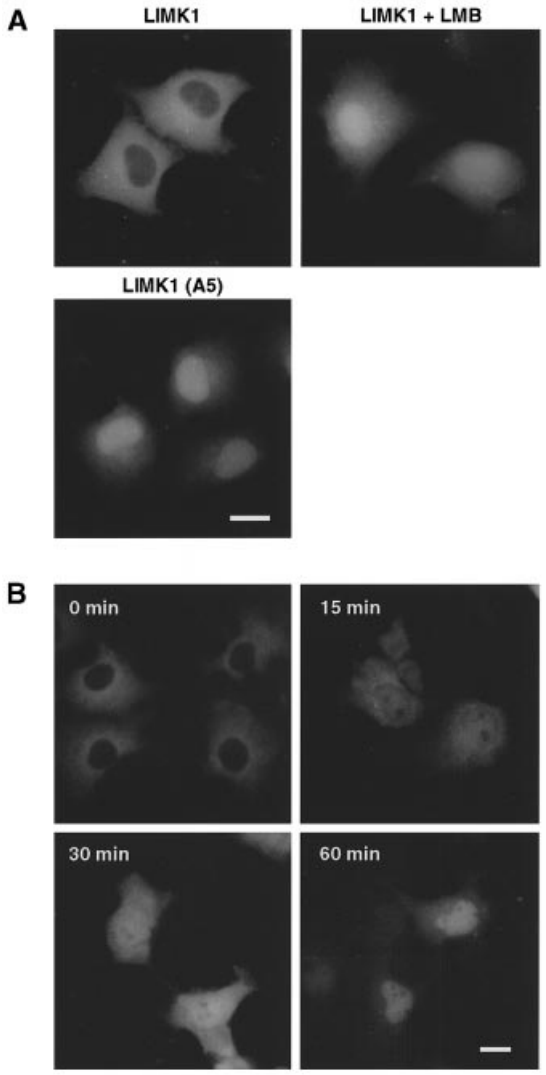

Figure 4 Nuclear accumulation of LIMK1 by disruption of two NESs or LMB treatment

(**A**) HeLa cells were transiently transfected with the plasmid coding for Flag-tagged full-length LIMK1 or LIMK1(A5). After 36 h, cells were fixed and stained with anti-Flag antibody. + LMB, HeLa cells transfected with Flag-LIMK1 were treated with LMB (4 ng/ml) for 60 min at 37 °C before fixation. (**B**) Time course of the nuclear accumulation of GFP–LIMK1 during LMB treatment. COS-7 cells were transiently transfected with the plasmid coding for GFP–LIMK1. After 16 h, cells were treated with LMB (4 ng/ml) and the localization of GFP–LIMK1 was observed by fluorescence microscopy at the indicated times. Scale bars, 20 μm.

LIMK1(A5ΔNLS), in which an NLS-like sequence (residues 496–504) was deleted from GFP–LIMK1(A5). As shown in Figure 5, when expressed in HeLa cells, GFP–LIMK1(A5) accumulated in the nucleus, whereas wild-type GFP–LIMK1 was almost completely localized in the cytoplasm. It is noted that GFP–LIMK1(A5ΔNLS), in contrast with GFP–LIMK1(A5), localized mainly in the cytoplasm, although a scant level of fluorescence was detected in the nucleus. These results suggest that the NLS-like sequence within the kinase domain is required for efficient accumulation of LIMK1(A5) in the nucleus and that this sequence most probably functions as an NLS for nuclear import of LIMK1.

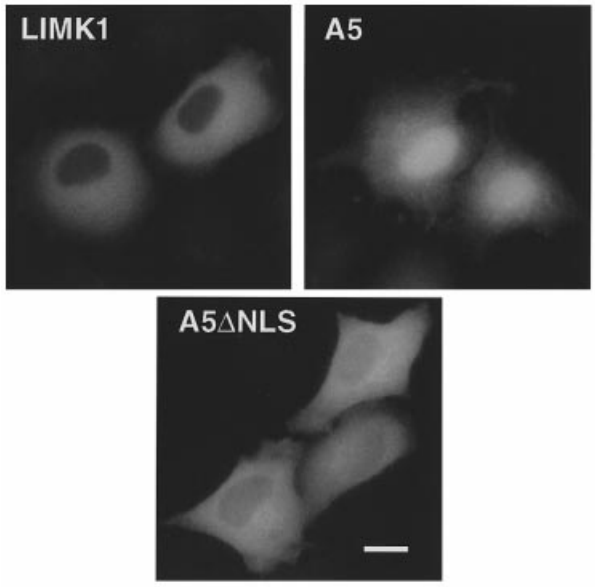

Figure 5 Nuclear accumulation of LIMK1(A5) is reduced by deletion of the NLS-like basic cluster sequence in the protein-kinase domain

HeLa cells were transfected with the expression plasmid coding for GFP–LIMK1, GFP–LIMK1(A5) or GFP–LIMK1(A5ΔNLS). After 16 h, localization of GFP-fusion proteins was visualized by fluorescence microscopy. Scale bar, 20 μm.

DISCUSSION

The nuclear import and export of relatively large proteins (with molecular masses > 40000–60000 Da) through nuclear pore complexes are generally selective and signal-dependent, and require both energy and soluble factors, including transport carrier proteins and a small GTPase, Ran [21–23]. In the present study we obtained evidence that LIMK1 contains two NESs within the PDZ domain that are similar to the previously characterized leucine-rich NESs [24–29]. Inhibition of the nuclear-export activity of LIMK1 by malfunctional mutation of these two NESs or LMB treatment induced change in the localization of LIMK1 from the cytoplasm to the nucleus. This finding indicates that the predominantly cytoplasmic localization of LIMK1, under normal conditions, is crucially supported by these two NESs. The NES-supported cytoplasmic localization is thought to be important primarily for the functions of LIMK1, since LIMK1 was shown to play a key role in regulation of actin-filament dynamics in the cytoplasm by phosphorylating cofilin [12,13].

On the other hand, the rapid accumulation of LIMK1 into the nucleus by LMB treatment (Figure 4B) indicates that LIMK1 normally shuttles between the cytoplasm and the nucleus. As the nuclear accumulation of LIMK1(A5) protein was prevented by deletion of a short basic cluster sequence within the kinase domain, the nuclear import of LIMK1 may well be mediated by this NLS-like short basic motif. Thus it is likely that LIMK1 shuttles continuously between the cytoplasm and the nucleus, utilizing two NESs in the PDZ domain and an NLS in the kinase domain. As the nuclear-cytoplasmic distribution of the shuttling protein is generally thought to be determined by the balance of rates of nuclear export and import, the apparently predominant localization of LIMK1 in the cytoplasm is probably supported by its rate of nuclear export being higher than its rate of nuclear

import. A similar result was noted for GFP-fusion protein coupled with both an NES of PKI-α and an NLS of simian virus 40 large-T antigen, which localized predominantly in the cytoplasm when expressed in yeast *Saccharomyces cerevisiae* [31]. Thus in these cases the NES activity seems to be more effective than the NLS activity.

The shuttling of LIMK1 between the cytoplasm and the nucleus may be the mechanism by which LIMK1 can rapidly alter the nucleocytoplasmic distribution in response to changes in cell conditions. When considering that LIMK1 may have a role in the nucleus, attention should be directed to a recent study suggesting that actin contains two NESs in the molecule and shuttles between the cytoplasm and the nucleus [27]. As cofilin contains an NLS in the sequence and can bind to actin, cofilin probably functions as a carrier for the nuclear import of actin [14,38]. Wada et al. suggested that the nucleocytoplasmic shuttling of actin is probably caused by the cofilin NLS-mediated nuclear import and the actin NES-mediated nuclear export [27]. Taking into account the findings that cofilin loses its actin-binding activity by phosphorylation at Ser-3 [14–17] and that LIMK1 catalyses this reaction [12,13], the nuclear LIMK1 may phosphorylate cofilin of the actin–cofilin complex imported into the nucleus, dissociate it to actin and phosphorylated cofilin, and hence promote the NES-mediated nuclear export of actin. This notion is based on the assumption that actin is imported into the nucleus as an actin–cofilin complex and is required to dissociate from cofilin for efficient nuclear export. Several types of stress, such as heat shock and treatment with DMSO, induce the translocation of actin and cofilin into the nucleus and the formation of actin–cofilin rod structures in the nucleus [39]. Although the physiological significance of stress-induced nuclear translocation and rod formation of actin and cofilin remains unknown, LIMK1 may perhaps be involved in these events by changing its nucleocytoplasmic distribution or activities in response to the stress.

We identified two NESs within the PDZ domain of LIMK1. The critical hydrophobic residues in these two NES sequences are not necessarily conserved in the corresponding sites of other PDZ domains. Thus, the nuclear-export activity of the PDZ domain of LIMK1 cannot be generalized to all, if any, of other PDZ domains. The PDZ domain has been thought to be a structural and functional unit, which is folded into a compact tertiary structure and functions as the protein-binding motif [11]. Some of the PDZ domains were shown to bind to the C-terminal S/TXV tripeptide motifs of transmembrane receptors and ion channels. These interactions are implicated in clustering of receptors and channels or in linking them to effector molecules, but others bound to distinct sequences [11,40]. Based on the three-dimensional structure predicted for the PDZ domain of the synaptic protein PSD-95 [41], NES-1 and NES-2 of LIMK1 locate in the αB and βF regions, respectively, regions distinct from the hydrophobic groove used for binding the C-terminal S/TXV peptides by the PDZ domain of PSD-95. Thus, it could be that the PDZ domains have the potential to exhibit multiple functions, using different surfaces of the domain. Although the PDZ domain of LIMK1 was not shown to bind to the S/TXV peptides that we tested previously [20], it may have the potential to bind to other sequences using the peptide-binding hydrophobic groove, in addition to the nuclear-export activity using two NESs.

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