Phosphorylation-dependent Regulation of Unique Nuclear and Nucleolar Localization Signals of LIM Kinase 2 in Endothelial Cells

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LIM kinases (LIMKs) regulate actin dynamics through cofilin phosphorylation and also have a function in the nucleus. Recently, we have shown that LIMK2 shuttles between cytoplasm and nucleus in endothelial cells and that nuclear import is inhibited by protein kinase C-mediated phosphorylation of Ser-283. Here, we aimed to identify the structural features of LIMK2 responsible for nuclear import. We found that the kinase domain of LIMK2 is localized exclusively in the nucleus and, in contrast to the kinase domain of LIMK1, it accumulated in the nucleolus. Through site-directed mutagenesis, we identified the basic amino acid-rich motif KKTRLKNDKRRK (amino acids 491–503) as the functional nuclear and nucleolar localization signal of LIMK2. After fusing this motif to enhanced green fluorescent protein, the fusion protein localized exclusively in the nucleus and nucleolus. Mutagenesis studies showed that phosphorylation of Thr-494, a putative protein kinase C phosphorylation site identified within the nuclear localization signal, inhibits nuclear import of the enhanced green fluorescent protein-PDZ kinase domain of LIMK2. After inhibiting nuclear export with leptomycin B, phosphorylation of either Ser-283 or Thr-494 reduced the nuclear import of LIMK2. Phosphorylation of both Ser-283 and Thr-494 sites inhibited nuclear import completely. Our findings identify a unique basic amino acid-rich motif (amino acids 491–503) in LIMK2 which is not present in LIMK1 that serves to target the protein not only to the nucleus but also to the nucleolus. Phosphorylation of Thr-494 within this motif negatively regulates nuclear import of LIMK2.

Endothelial cell structure and functional integrity are important in the maintenance of the vessel wall and circulatory function. Contraction, migration, and proliferation of vascular endothelial cells control vascular permeability, endothelial repair after injury, and angiogenesis (1, 2). The LIM kinases (LIMKs), consisting of LIMK1 and LIMK2, are serine/threonine protein kinases that regulate the actin dynamics via phosphorylating the actin-depolymerizing protein cofilin (3, 4). Besides this, various studies showed that LIMKs may have a function in the nucleus (5, 6). The phenotype of LIMK2 knock-out mouse showed a defect in spermatogenesis, suggesting a nuclear function of tLIMK2, a testis-specific LIMK2 splice form lacking both LIM domains and being preferentially localized in the nucleus (7). It has been shown that the nuclear localization of LIMKs can mediate suppression of Rac/Cdc42-mediated cyclin D1 expression. This effect of LIMKs was independent of cofilin phosphorylation and the regulation of actin dynamics (8).

LIMK1 and LIMK2 are localized predominantly in the cytoplasm but accumulate in the nucleus when the cells are treated with the chromosomal region maintenance 1-dependent export inhibitor leptomycin B (LMB), suggesting that these kinases contain nuclear localization signals (NLS) (9, 10). NLSs are often characterized by clusters of basic amino acids. The main types of NLS known are monopartite and bipartite NLSs (11). The classical monopartite NLS is composed of a single cluster of basic amino acids such as SV40 large T-antigen NLS (PKKKRKV). The bipartite NLS comprises two clusters of basic amino acids separated by a 10–12-amino acid spacer such as the NLS of nucleoplasmin (KRPAAKKAGQAKKKKLDK) (11, 12). The kinase domain of LIMKs has a unique basic amino acid-rich motif between subdomains VII and VIII (13). The basic nature of this motif suggests that it may function as a NLS (9). Moreover, LIMK1 has two nuclear export signal (NES) sequences within the PDZ domain (14). These structural features suggest that LIMKs can actively shuttle between the cytoplasm and nucleus. However, the NLS and NES of LIMK2 have not been characterized.

We have recently shown that nucleocytoplasmic shuttling of LIMK2 in endothelial cells is regulated by PKC activation. We could demonstrate that Ser-283 phosphorylation of LIMK2 by PKC inhibits its nuclear import (10). In the present study we identified a unique basic amino acid-rich motif (491–503) in LIMK2 that is not present in LIMK1. Interestingly, this motif of LIMK2 served to target the EGFP protein not only to the nucleus but also to the nucleolus.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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2 The abbreviations used are: LIMK, LIM kinase; aa, amino acids; EGFP, enhanced green fluorescent protein; PDZK, EGFP-PDZ kinase domain construct of LIMK2; NLS, nuclear export signal; NLS, nuclear localization signal; NoLS, nucleolar localization signal; LIM domain, acronym for lim, isl, mec domain; LMB, leptomycin B; PDZ, PSD-95, disc large, ZO-1; PKC, protein kinase C; HIV, human immunodeficiency virus.
nucleus but also to the nucleolus. Phosphorylation of Thr-494 within this motif reduced the nuclear import of LIMK2, and phosphorylation of both Ser-283 and Thr-494 inhibited the nuclear import of LIMK2 completely.

EXPERIMENTAL PROCEDURES

Materials—LMB was from Sigma. Oligonucleotides were synthesized by MWG Biotech AG (Ebersberg, Germany).

Construction of the Expression Plasmids and Site-directed Mutagenesis—The pUC-SRα-LIMK2 vector containing full-length cDNA of LIMK2 was kindly provided by Prof. K. Mizuno (Tohoku University, Sendai, Japan). The full-length coding sequence of LIMK2 was amplified by PCR using pUC-SRα-LIMK2 as a template. The PCR-amplified product was cloned into EcoRI and Sall sites of pEGFP-C1 vector (BD Biosciences Clontech) to obtain LIMK2 fused with EGFP. The full-length cDNA of LIMK1 was amplified by PCR from a cDNA pool of human umbilical vein endothelial cell total RNA. The following constructs of LIMK2 and LIMK1 were cloned into EcoRI and Sall sites of pEGFP-C1 vector: EGFP-NLS (aa 475–510) EGFP-PDZ kinase (aa 142–638, PDZK) and kinase domain (aa 315–638) of LIMK2, and kinase domain (aa 302–647) of LIMK1. The mutants of pEGFP-PDZ kinase and pEGFP kinase were generated by QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) as per the manufacturer’s instructions. All the constructs were confirmed by DNA sequencing (Agowa GmbH Berlin, Germany).

Cell Culture and Transfection—Human umbilical vein endothelial cells were obtained and cultured as described previously (15). Briefly, cells harvested from umbilical cords were plated onto collagen-coated (room temperature, 75 μg/ml collagen G; Biochrom, Berlin, Germany) plastic culture flasks and were cultured in complete endothelial growth medium (Promo Cell, Heidelberg, Germany) at 5% CO2 and 37 °C. In all experiments, human umbilical vein endothelial cells up to the third passage were used.

Transient transfection of endothelial cells was performed by electroporation as previously described (10). Briefly, cells were grown up to 90% confluency, harvested by trypsin/EDTA (Sigma) treatment, and washed with phosphate-buffered saline. Cells (1.4 × 10⁶ cells/400 μl) were resuspended in electroporation buffer (20 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM D-glucose, pH 7.0). Plasmids (20 μg in 30–60 μl of electroporation buffer) were mixed with the cell suspension, and cells were incubated for 10 min (room temperature) then transferred into a 4-mm-gap electroporation cuvette for electroporation at 1000 microfarads.
and 210 V (Bio-Rad Gene Pulser). Thereafter, 400 μl of complete endothelial growth medium (without antibiotics) was added in the cuvette. Cells were then transferred to collagen-coated glass-bottom Petri dishes that are designed for confocal microscopy (MatTek Corp., Ashland, MA) and grown in fresh complete endothelial growth medium for 24 h (washing and replenishment after 1 and 12 h). The transfection efficiency was 30–60%.

Confocal Microscopy—After 24 h of transfection, cells were observed with a Zeiss LSM510 confocal laser-scanning microscope. In some experiments cells were treated with LMB (10 ng/ml) for 60 min to inhibit chromosomal region maintenance 1-mediated nuclear export. Cells were kept under the microscope at 37 °C. An argon laser (488 nm) was used as a light source for EGFP excitation. The microscope function was controlled by a light manager through the software LSM 510 META. For Z-stacking, the top and the bottom position were selected, and 8–15 slices were determined according to the pinhole size and scanning time. The area and the mean intensity of the EGFP were measured using the LSM 510 META software. The measurements were carried out in three independent experiments with 20 cells randomly selected in each experiment. The mean ± S.E. was calculated for each experiment.

RESULTS

Prediction of Nuclear Localization Signals in LIMK2—Our previous study showed that the deletion of both LIM domains of LIMK2 but not LIMK1 enhanced the nuclear accumulation of the truncated protein (10). These data indicate that LIMK2 localized more efficiently to the nucleus perhaps due to the differences in the NLS compared with LIMK1. Both LIMK1 and LIMK2 have a unique basic amino acid-rich region in the kinase domain. LIMK2 has a second basic amino acid-rich motif between the PDZ and the kinase domains (Fig. 1 A). To predict the functional NLS in LIMK2, the basic amino acid-rich motifs of LIMK2 were analyzed by searching the Prosite data base and by manual comparison with the known NLSs of different proteins (Fig. 1, B and C, and Ref. 16). Four unique potential NLSs were predicted in LIMK2, which were designated as NLS1, NLS2, NLS3, and NLS4 (Fig. 1D), whereas only one monopartite NLS was found in LIMK1 (Fig. 1B).

The monopartite NLS1 in LIMK2 and the NLS of LIMK1 were predicted by comparing these motifs with well characterized monopartite NLSs of different proteins (see Fig. 1B and Ref. 17). NLS3 of LIMK2, a classical bipartite NLS, was predicted by searching the
Prosite data base (Fig. 1D). The bipartite NLS2 was unraveled by aligning the Polo-like kinases with the basic amino acid motifs of LIMK2 (18). In contrast to NLS3, NLS2 has a short spacer sequence of only 6-amino acids between two clusters of basic amino acids (Fig. 1C). Of interest, within the 6-amino acid spacer of NLS2, two further basic amino acids (RK) are present. NLS4 was predicted as a NLS due to a cluster of five basic amino acids.

Beside four NLSs, two NESs were predicted in LIMK2 based on alignment with LIMK1 and the consensus sequence of NES (ψX$_2$-ψX$_2$-LX(L/I)), where ψ represents any hydrophobic amino acid). LIMK1 has two leucine-rich nuclear export signals (NES1 and NLS2) in the PDZ domain (9). The putative NES1 of the LIMK2 is present at the C terminus of PDZ domain, and NSE2 is localized immediately after the PDZ domain (supplemental Table 1).

The Kinase Domain of LIMK2 Localizes to the Nucleus and the Nucleolus—EGFP-PDZK kinase of LIMK2 (EGFP-PDZK) contains all the predicted NESs and NLSs. Indeed, we have previously shown that PDZ kinase of LIMK2 (amino acids 142–638) was localized both in the nucleus and the cytoplasm (10). The kinase domain of LIMK2 containing three predicted NLSs but no NES should localize mainly in the nucleus. To verify this prediction, the kinase domain (amino acids 315–638) fused with EGFP was transfected into endothelial cells. It was found that more than 95% of EGFP kinase accumulated in the nucleus (Fig. 2A). EGFP alone accumulated only to 42% in the nucleus (data not shown). Notably, EGFP kinase was also concentrated as dense bodies in the nucleus (white arrow, Fig. 2A), indicating that EGFP kinase might associate with specific nuclear subcompartments, such as the nucleolus, the Cajal bodies, and nuclear speckles. Of these subcompartments, the nucleolus is a highly dense nuclear compartment that can be easily detected by phase contrast microscopy. Phase contrast images of EGFP kinase-transfected endothelial cells showed an exact overlap of nucleoli with intensely stained EGFP kinase dense bodies (Fig. 2A, arrowhead), indicating that the EGFP kinase of LIMK2 not only accumulates in the nucleus but also in the nucleolus. In contrast, EGFP kinase of LIMK1 did not localize to the nucleolus (Fig. 2A, arrows and arrowheads). These results suggest that the kinase domain of LIMK2, but not LIMK1, has a specific nucleolar localization signal (NoLS).

Identification of Nucleolar and Nuclear Localization Signals of LIMK2 by Site-directed Mutagenesis of the Kinase Domain—NoLS are sequences mostly rich in arginine and lysine. They often overlap with NLSs. No specific consensus sequences for nucleolar localization have been determined (19–21).

To analyze the role of various basic amino acid-rich stretches in the kinase domain of LIMK2 (aa 480–503) for nucleolar and nuclear localization, different mutants of EGFP kinase were generated in which the basic amino acids were mutated to alanines. The subcellular localization of the EGFP kinase mutants was then studied in endothelial cells. The mutants generated are shown in Table 1.

Mutant 1 (NLS1, RKKR, aa 500–503), mutant 2 (RK, aa 496–497), mutant 3 (KKR, aa 491–493), and mutant 4 (RK, amino acids 480–482) were mainly localized in the nucleus similar to the wild type kinase domain of LIMK2. However, the nucleolus staining was drastically reduced (Fig. 2B, Table 2). These results indicate that NLS1 (RKKR) and the basic amino acid clusters RK and KKR are important parts of the NoLS. In contrast, the nucleolar localization of mutant 4 was only slightly reduced (Fig. 2B, g and h). These results indicate that each of the basic amino acid-rich clusters within the motif 491–503 (KKRTL-RKNDRKKR) is required for nucleolar localization but not for nuclear localization. The basic amino acids 480–482 play only a minor role in nucleolar localization.

The nuclear localization of the double mutants, mutant 5 (RK and RKKR), mutant 6 (NLS2, KKR, and RKKR), and mutant 7 (NLS 3, RK, and KKR) was decreased by only 25%. These mutants were also absent in the nucleolus (Fig. 2B, Table 2). When all the basic amino acids within the motif (aa 491–503) were mutated to alanines (mutant 8), the nuclear localization of EGFP kinase was drastically reduced (Table 2; 20 ± 4%), and its nucleolar localization was absent. These results indicate that NLS2 containing the basic amino acids RK within the spacer region (amino acids 491–503) is the NLS of LIMK2.

**NLS4 Is Not a Functional Nuclear Localization Signal**—To explore whether NLS4 between the PDZ and the kinase domains can function as a nuclear localization signal, the kinase

### TABLE 1

| Mutant of EGFP kinase | Amino acid sequence |
|-----------------------|---------------------|
| Wild type             | RKR->PMEKATTTLRKR(R) |
| Mutant 1 (NLS1)       | RKR->PMEKATTTLRKR(R) |
| Mutant 2              | RKR->PMEKATTTLRKR(R) |
| Mutant 3              | RKR->PMEKATTTLRKR(R) |
| Mutant 4              | RKR->PMEKATTTLRKR(R) |
| Mutant 5              | RKR->PMEKATTTLRKR(R) |
| Mutant 6 (NLS2)       | RKR->PMEKATTTLRKR(R) |
| Mutant 7 (NLS3)       | RKR->PMEKATTTLRKR(R) |
| Mutant 8 (NLS2 + RK)  | RKR->PMEKATTTLRKR(R) |

### TABLE 2

**Summary of the mutation data of EGFP-kinase**

The subcellular distribution of EGFP-kinase was measured as described under “Experimental Procedures” and calculated as % of total expressed recombinant protein. The values of subcellular expression of the recombinant protein are the mean ± S.E. (n = 3 experiments, 20 cells per experiment were selected). N versus C indicates the nuclear localization versus cytoplasmic localization of the EGFP-kinase (> or < represents 20–25% of total expressed protein).

| Name of the mutant | Sub-cellular localization |
|-------------------|--------------------------|
|                   | Nucleus | N versus C | Nucleus |
| Wild type EGFP-kinase | 95 ± 2 | N>>><>C | Yes |
| Mutant 1          | 85 ± 4 | N>>><>C | No |
| Mutant 2          | 80 ± 6 | N>>><>C | No |
| Mutant 3          | 80 ± 6 | N>>><>C | No |
| Mutant 4          | 90 ± 1 | N>>><>C | Yes |
| Mutant 5          | 75 ± 2 | N>>><>C | No |
| Mutant 6          | 75 ± 1 | N>>><>C | No |
| Mutant 7          | 75 ± 5 | N>>><>C | No |
| Mutant 8          | 20 ± 4 | N<<<<C | No |
domain-deleted LIMK2 construct (EGFP-Dkinase-LIMK2; aa 1–314) was prepared. The EGFP-Dkinase-LIMK2 protein was localized in the cytoplasm and not in the nucleus. The two putative chromosomal region maintenance 1-dependent NESs of LIMK2 present in the EGFP-Dkinase-LIMK2 (supplemental Table 1) may be responsible for its localization in the cytoplasm. To inhibit the NES functions, cells were treated with LMB. After 1 h of treatment, EGFP-Dkinase-LIMK2 protein was distributed equally in the nucleus and the cytoplasm, suggesting a partial role of NLS4 for nuclear localization (Fig. 3A).

To analyze further, the basic amino acids 280–282 (RRR) were mutated to alanine in EGFP-Dkinase-LIMK2. The subcellular distribution of the mutant protein was, however, similar to that of the wild type construct in cells after LMB treatment (Fig. 3A). These results indicate that NLS4 is not functional. After LMB treatment, the presence of the EGFP-Dkinase-LIMK2 protein in the nucleus might be explained by the passive diffusion of this low molecular weight protein (60 kDa) into the nucleus.

Analysis of the NLS by Site-directed Mutagenesis of the PDZK Domain of LIMK2—In comparison with the lower molecular mass EGFP kinase (∼62 kDa) protein, the EGFP-PDZK (∼84 kDa) is expected to be less in the nucleus, since it is unlikely to be transported into the nucleus by passive diffusion and contains two putative NESs. To analyze whether the NLS motif identified in the kinase domain (aa 491–503) is also sufficient for nuclear localization of EGFP-PDZK of LIMK2, selected mutants were studied (Table 1).

EGFP-PDZK was localized in the cytoplasm and nucleus. In 25% of the cells, nucleolar staining was observed. All the mutants of EGFP-PDZK were mainly localized in the cytoplasm (Table 3 and Fig. 3B). After LMB treatment, nuclear localization of mutant 9 (affecting NLS1) and mutant 11 (affecting NLS2) was only 20% (Fig. 3B, Table 3) indicating

![FIGURE 3. The basic amino acid-rich motif (491–503) but not NLS4 is a functional nuclear and nucleolar localization signal of LIMK2.](image)

**TABLE 3**

Summary of the mutation data of EGFP-PDZK

| Name of the mutant | Sub-cellular localization | Control | +LMB | Nucleolus |
|--------------------|---------------------------|---------|------|-----------|
| Wild type EGFP-PDZK| 40 ± 5                    | 100     | Yes  |
| Mutant 9           | 2 ± 2                     | 20 ± 5  | No   |
| Mutant 10          | 2 ± 2                     | 40 ± 6  | No   |
| Mutant 11          | 2 ± 2                     | 20 ± 5  | No   |
| Mutant 12          | 2 ± 2                     | 7 ± 3   | No   |

The sub-cellular distribution of EGFP-PDZK was measured before and after LMB treatments described under "Experimental Procedures" and calculated as % of total expressed recombinant protein. The values of sub-cellular expression of the recombinant protein are the mean ± S.E. (n = 3 experiments, 20 cells per experiment were selected). N/C indicates the nuclear localization versus cytoplasmic localization of the EGFP-PDZK.
that amino acids 491–493 (KKR) and 500–503 (RKKR) are the major parts of the functional NLS. Nuclear localization of mutant 10 (affecting RK, 496–497) was higher (40%) than of mutants 9 and 11 (Fig. 3B, Table 3), indicating that these two basic amino acids are also a part of the NLS of LIMK2. When all the basic amino acids of the identified NLS (Mutant 12) were mutated to alanine, the mutant protein was exclusively in the cytoplasm (98%; Fig. 3B, Table 3), further supporting that the identified NLS motif (aa 491–503) is the nuclear localization signal of LIMK2. None of the mutants was observed in the nucleolus, indicating that all the basic amino acids of the motif (491–503) are required for the nuclear localization of LIMK2.

**The NLS of LIMK2 Is Sufficient to Translocate EGFP into the Nucleus and Nucleolus**—To determine whether the identified NLS motif could transport EGFP in the nucleus and nucleolus, the amino acid sequence 475–510 of LIMK2 was fused to the C terminus of EGFP. The new fusion protein (EGFP-NLS) was completely localized to the nucleus and nucleolus (Fig. 3C), proving that the identified motif is the functional NLS and NoLS of LIMK2.

**Phosphorylation of Thr-494 Reduces Nuclear Import of LIMK**—One putative PKC phosphorylation site (Thr-494) is present within the identified NLS, and it might affect the nuclear import after phosphorylation. When we studied the phospho-mimicking mutant of PDZK-LIMK2 (T494EE) in endothelial cells, we found that this mutant was exclusively present in the cytoplasm of resting endothelial cells (Fig. 4A). After LMB treatment, the mutant protein was partially enriched in the nucleus but was still less as compared with the wild type PDZK of LIMK2 (compare Fig. 4A with Fig. 3B). These results indicate that Thr-494 phosphorylation inhibits the NLS function of LIMK2; however, it is insufficient to block nuclear import completely.

**Phosphorylation of Ser-283 Together with Thr-494 Phosphorylation Inhibits Nuclear Import Completely**—Previously we have shown that the PKC-dependent phosphorylation of LIMK2 at Ser-283 inhibited nuclear import, but the S283EE mutant protein still could enter the nucleus after LMB treatment (10). We asked whether the phosphorylation of the two PKC sites can block the nuclear import completely. To answer this question, we prepared several double mutants of EGFP-PDZK of LIMK2. After LMB treatment, S283EE/T494A (active/inactive) mutant and S283A/T494EE (inactive/active) mutant behaved similar to the single active mutants S283EE and T494EE, respectively; they were partially enriched in the nucleus (Fig. 4, A and B) (10). The double active mutant (S283EE/T494EE) of EGFP-PDZK and EGFP-LIMK2 were localized exclusively in the cytoplasm and were unable to localize in the nucleus of endothelial cells after
LMB treatment (Fig. 4, B and C). These results indicate that phosphorylation of either one of the two PKC sites is not sufficient to inhibit the nuclear import completely and that phosphorylation of both sites is required to block the nuclear import of LIMK2 completely.

**DISCUSSION**

In this study we describe two important structural features of LIMK2 that regulate its nuclear import; we identified the motif KK503 as NLS as well as NoLS, and we found a putative PKC phosphorylation site within this motif (Thr-494) that partially inhibits nuclear import of LIMK2 in endothelial cells. We showed that the function of this motif was to target proteins not only to the nucleus but also to the nucleolus; fusion of a short stretch of amino acids containing this motif with EGFP lead to the localization of EGFP into the nucleus and nucleolus. Of note, our mutagenesis studies showed that the 4th basic amino acid cluster (aa 480–482) was not important in targeting the kinase and PDZ kinase domain of LIMK2 to the nucleus and nucleolus. Also, another possible NLS (NL54) between the PDZ and kinase domains of LIMK2 was not functional.

We have identified a putative PKC phosphorylation site (Thr-494) within the NLS of LIMK2 that inhibits nuclear import. Many nucleocytoplasmic shuttling proteins such as diacylglycerol kinase ζ, Ca²⁺/calmodulin-dependent protein kinase II, and cyclin B1 are phosphorylated near their NLS, thereby affecting their affinity to the importin protein complex (17, 22–24). Our previous study showed that PKC activation inhibits nuclear import of LIMK2 by phosphorylating the kinase at Ser-283. Our present data indicate that Ser-283 and Thr-494 phosphorylation cooperate in inhibiting the nuclear import of LIMK2.

The identified functional NLS is not a classical monopartite and bipartite NLS. Unlike the classical bipartite NLS consisting of a defined spacer of 8–10 non-basic amino acids between two clusters of basic amino acids, the NLS and NoLS of LIMK2 is made of three clusters of basic amino acids. In contrast to LIMK2, LIMK1 has only an 8-amino acid (499–506) motif with two clusters of basic amino acids, suggesting different functions of the NLS of LIMK1 and LIMK2. Indeed, the kinase domain of LIMK2 but not LIMK1 was present in the nucleolus.

Unlike the nucleus, there is no evidence for the existence of a barrier separating the nucleolus from the surrounding nucleoplasm. As a consequence, any soluble molecule should in principle diffuse in and out of the nucleolar compartment. Sequence analysis of nucleolar proteins did not lead to the identification of a general NoLS (25–27). Nucleolar localizing properties of proteins were apparently due to cooperation of several domains, suggesting a complex regulation of nucleolar localization (28, 29). However, small NoLS sequence motifs have been identified mainly in retroviral RNA binding trans-regulator proteins. Aligning the LIMK2 NoLS with the published NoLS sequences (generally 20–40 amino acids long) (30–32) revealed that all these sequences retain continuous stretches of basic residues; either one continuous stretch of three to four basic residues or one to three stretches of three to four basic residues interrupted by one non-basic residue (Table 4). However, each of these basic amino acids clusters was shown to be important but not sufficient for nucleolar localization of the protein (30, 31). We also found by site-directed mutagenesis that each of the basic amino acid clusters (aa 491–503) was important for nucleolar localization of the kinase domain of LIMK2. Moreover, we could demonstrate that the sequence containing all three clusters of basic amino acids was sufficient to target EGFP to the nucleolus. Thus, this sequence functions as a NoLS.

Targeting of the LIMK2 to the nucleolus might be accomplished by one of three different types of interactions; (a) with nucleolar or nucleolar-associated proteins and (b) with rDNA and (c) nucleolar RNA consisting mainly of rRNA (33, 34). Arginine/lysine-rich RNA binding domains are present in many viral proteins that associate with the nucleolus (35–38). These proteins have a similar pattern of basic amino acid distribution as in LIMK2, suggesting that LIMK2 may bind to rRNA (Table 5). The binding of proteins to rRNA in the nucleolus is known to regulate ribosome biogenesis necessary for cell division and gene transcription (27, 39).

Whether the nuclear and nucleolar function of LIMK2 involves its interaction with actin and cofilin remains to be explored. Recent studies showed that actin and associated proteins are found in various sub-compartments of the nucleus such as the nucleolus and speckles (40, 41) and that actin is involved in chromatin remodeling and gene transcription (41, 42).

**Acknowledgments**—The technical assistance of Nicole Wilke is greatly appreciated. We thank Prof. Michael Schleicher for providing access to confocal microscopy.

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**TABLE 4**

| Protein | Sequence |
|---------|----------|
| Werner, 1027–1058 | FLVEVSRNYKMKICALT)|
| Nucleolin, 691–714 | FRGQGGGCGGGDFPEQQPEFKKFR |
| La, 323–354 | QSELMWKSKRREPCGKKGGK |
| L5, 240–271 | YKKKKAKKRPLKPRKPRKPRKPR |
| IGF-I, 161–195 | GTELEQIQEKKKQQKKEQKKEKKEK |
| HIV-1 Rev, 35–50 | RQKRRR KKRRKRRKRR |
| HTLV-I Rev, 2–19 | PFPKPRRR RRKPRRPP |
| LIMK2, 491–503 | KKRTLRRR KRRR |

**TABLE 5**

Comparison of the arginine/lysine-rich RNA binding domains of different nucleolar proteins with the NoLS sequence of LIMK2

Basic amino acids essential for RNA binding are shown in boldface and are underlined.
Nucleolar and Nuclear Localization of LIMK2

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