Inhibition of Nuclear Import of LIMK2 in Endothelial Cells by Protein Kinase C-dependent Phosphorylation at Ser-283

Pankaj Goyal‡, Dharmendra Pandey, Antje Behring, and Wolfgang Siess§

From the Institute for Prevention of Cardiovascular Diseases, University of Munich, 80336 München, Germany

LIM kinases (LIMKs) are mainly in the cytoplasm and regulate actin dynamics through cofilin phosphorylation. Recently, it has been reported that nuclear localization of LIMKs can mediate suppression of cyclin D1 expression. Using immunofluorescence monitoring of enhanced green fluorescent protein-tagged LIMK2 in combination with photobleaching techniques and leptomycin B treatment, we demonstrate that LIMK2 shuttles between the cytoplasm and the nucleus in endothelial cells. Sequence analysis predicted two PKC phosphorylation sites in LIMK2 but not in LIMK1. One site at Ser-283 is present between the PDZ and the kinase domain, and the other site at Thr-494 is within the kinase domain. Activation of PKC by phorbol ester treatment of endothelial cells stimulated LIMK2 phosphorylation at Ser-283 and inhibited nuclear import of LIMK2 and the PDZ kinase construct of LIMK2 (amino acids 142–638) but not of LIMK1. The PKC-ε isoform phosphorylated LIMK2 at Ser-283 in vitro. Mutational analysis indicated that LIMK2 phosphorylation at Ser-283 but not Thr-494 was functional. Serum stimulation of endothelial cells also inhibited nuclear import of PDZK-LIMK2 by protein kinase C-dependent phosphorylation of Ser-283. Our study shows that phorbol ester and serum stimulation of endothelial cells inhibit nuclear import of LIMK2 but not LIMK1. This effect was dependent on PKC-ε-mediated phosphorylation of Ser-283. Since phorbol ester enhanced cyclin D1 expression and subsequent G1-to-S-phase transition of endothelial cells, we suggest that the PKC-mediated exclusion of LIMK2 from the nucleus might be a mechanism to relieve suppression of cyclin D1 expression by LIMK2.

Contraction, migration, and proliferation of vascular endothelial cells are essential features of vascular permeability, endothelial repair after injury, and angiogenesis (1, 2) and are regulated by coordinated changes of actin dynamics (3, 4). The LIMK family of proteins, a member of the class of serine/threonine protein kinases, consists of LIMK1 and LIMK2 that specifically phosphorylate and inactivate cofilin, an actin-depolymerizing protein, thereby regulating actin cytoskeleton rearrangement (5, 6). Other studies showed that cell cycle progression depends on the regulated activity of the LIMK-cofilin system (7, 8). The kinase activity of LIMKs is regulated by members of the RhoGTPase family, Rho, Rac, and Cdc42, via their downstream protein kinases Rho kinase and p21-activated kinases 1 and 4. These kinases phosphorylate LIMK1 at Thr-508 in the activation loop of the kinase domain (9–11). Rho kinase activates LIMK2 by phosphorylation at Thr-505 (12, 13).

Several lines of evidence suggest that LIMKs also have a function in the nucleus. LIMK1 is predominantly localized in the cytoplasm but accumulates in the nucleus, when the cells are treated with the CRM1-dependent export inhibitor, leptomycin B (LMB) (14). In mouse tissues, various splice forms of LIMK2 have been reported that display an unique cellular localization (15, 16). LIMK2a (full-length) and LIMK2b containing only one LIM domain are localized exclusively in the cytoplasm (17). In contrast, tLIMK2 (testis-specific LIMK2 splice form) lacking both LIM domains was found to be preferentially localized in the nucleus (18). The phenotype of the LIMK2 knock-out mouse showed a defect in spermatogenesis, suggesting a nuclear function of tLIMK2 in testis (18).

The kinase domain of LIMKs has a unique basic amino acid-rich motif between subdomains VII and VIII. The basic nature of this motif suggests that this may function as a nuclear localization signal (NLS). Moreover, LIMK1 has two export signal sequences within the PDZ domain (14). The NLS and NES might explain the nucleocytoplasmic shuttling of LIMK1. A recent study shows 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 regulation of actin dynamics (19).

In the present study, we addressed the question of whether LIMK2 shuttles between the nucleus and the cytoplasm in endothelial cells and explored possible mechanisms of regulation of LIMK2 nucleocytoplasmic shuttling. We found that LIMK2 shuttles between the cytoplasm and the nucleus in endothelial cells and discovered that PKC inhibits the nuclear import of LIMK2 by phosphorylating the enzyme at Ser-283. PKC-mediated exclusion of LIMK2 from the nucleus might relieve suppression of cyclin D1 expression, leading to G1 phase cell cycle progression.
PKC Inhibits Nuclear Import of LIMK2

**Materials**—Phorbol 12-myristate 13-acetate (PMA) and LMB were from Sigma. The recombinant PKCs and the PKC inhibitors Go6976 and Go6983 were from Merck Biosciences GmbH. Anti-cofilin antibody (AFC1L2) was from Cytoskeleton (Denver, CO). Anti-phospho-cofilin (Ser3) and anti-phospho-Ser 186-LIMK2 substrate antibodies were from Cell Signaling Technology (Beverly, MA). Anti-EGFP antibody was kindly gifted by Dr. Hans Faix, (Institute of Cell Biology, University of Munich, Munich, Germany). Protein A-Sepharose (P-3391) was from Sigma. Oligonucleotides were synthesized by MWG Biotech AG (Ebersberg, Germany). The Phochepex kit was a gift from ProteinTech (Beverly, MA). Anti-EGFP antibody was kindly gifted by Dr. Hans Faix, (Institute of Cell Biology, University of Munich, Munich, Germany). Protein A-Sepharose (P-3391) was from Sigma. Oligonucleotides were synthesized by MWG Biotech AG (Ebersberg, Germany).

**Construction of the Expression Plasmids**—The pUC-Sr0-LIMK2 vector containing full-length cDNA of LIMK2 was kindly provided by Prof. Mizuno (Tohoku University, Sendai, Japan). The full-length coding sequence of LIMK2 was amplified by PCR with pUC-Sr0-LIMK2 as a template. The PCR-amplified product was cloned into EcoRI and SalI sites of pEGFP-C1 vector (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 SalI sites of pEGFP-C1 vector: ΔLIM1-LIMK2 (amino acids 69–638), ΔLIM2-LIMK2 (amino acids 72–124 deleted in full-length), PDZ kinase (amino acids 142–638, PDZK), and kinase domain (amino acids 315–638) of LIMK2 and PDZ kinase (amino acids 146–647, PDZK1) of LIMK1. The mutants of pEGFP-PDZ kinase (S283A, S283EE, T494A) were generated in the parent LIMK2 full-length expression kit (La Jolla, CA) as per the manufacturer’s instructions. To express recombinant His-tagged LIMK2 in *P. pastoris*, the PCR-amplified full-length LIMK2 was cloned into pPICZ expression vector by using EcoRI and NotI sites. All the constructs were confirmed by DNA sequencing (Agowa GmbH Berlin, Germany).

**Expression of Recombinant LIMK2 in *P. pastoris***—Expression plasmid pPICZ-LIMK2 was electroporated in *P. pastoris* strain GS115, and the cells with the highest protein expression was selected as per the manufacturer’s instructions. The selected colony of transformed cells was grown in 300 ml of BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 7.0, 1.34% (w/v) yeast nitrogen base, 4 x 10^{-5} (w/v) biotin, and 1% (w/v) glycerol). After 30–36 h of incubation at 28 °C, the cells were pelleted at 1000 x g and were resuspended in BMMY medium (BMGY medium in which the glycerol was replaced by 1% (v/v) methanol) to induce protein expression, and the cells were harvested 24 h later by centrifugation at 3000 x g at 4 °C for 10 min. The cells were washed once with ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 10% glycerol) and resuspended in breaking buffer supplemented with protease inhibitor mixture. An equal volume of acid-washed chilled glass beads (0.5 mm in diameter) was added to the samples, and cells were disrupted by vortexing for 10 times for 1 min, with intervening 1 min of incubation on ice. Cell debris were removed by centrifugation at 2000 x g at 4 °C for 5 min followed by ultracentrifugation at 75,000 x g for 10 min. The clear supernatant was bound to the pre-equilibrated Protein Ni 2000 pre-packed (Macherey-Nagel) poly-histidine tag purification column at 4 °C, and His-tagged LIMK2 was purified according to the manufacturer’s instructions.

**Cell Culture and Transfection**—Human umbilical vein endothelial cells were obtained and cultured as described previously (20). 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 at 5% CO2 and 37 °C in complete endothelial growth medium (PromoCell, Heidelberg, Germany). In all experiments, human umbilical vein endothelial cells up to third passage were used.

**Transient transfection of endothelial cells was performed by electroporation** as described previously (21) with modifications. Briefly, cells were grown up to 90% confluency, harvested by trypsin/EDTA (Sigma) treatment, and washed with phosphate-buffered saline. Cells (1 x 10^6 cells/400 µl) were suspended in electroporation buffer (20 mM Hepes, 137 mM NaCl, 0.7 mM EDTA, pH 7.5), 0.1% immuno-precipitates were further processed for immunoblot analysis. The cell suspension. The cells were incubated for 10 min (room temperature) and then transferred into a 4-mm gap electroporation cuvette. Cells were electroporated at 1000 microfarads and 210 V (Bio-Rad Gene Pulser). 400 µl of complete endothelial growth medium (without antibiotics) was added, and cells were transferred to collagen-coated glass bottom Petri dishes that are designed for confocal microscopy (MatTek Corp., Ashland, MA). The cells were grown in fresh complete endothelial growth medium for 24 h to obtain subconfluency (washing and replenishment after 1 and 12 h). The transfection efficiency was 60%.

**Immunoprecipitation**—Unstimulated and PMA-stimulated endothelial cells were washed with ice-cold phosphate-buffered saline and lysed in an equal volume of 2 x immunoprecipitation lysis buffer (2% Nonidet P-40, 300 mM NaCl, 20 mM Tris (pH 7.5), 2 mM EGTA, 2 mM EDTA, 5 mM Na3VO4, Complete mini protease inhibitor, 1 tablet/5 ml (Roche Applied Science), phosphatase mixture 1:100, and 0.1% SDS) for 45 min on ice. The lysates were clarified by centrifugation at 16,000 x g for 15 min, and then 40 µl of 50% protein A-Sepharose slurry was added to the supernatants and incubated for 1 h at 4 °C to preclar the supernatant. Protein A-Sepharose was prepared by incubating the beads in swelling buffer (20 mM NaH2PO4, 0.15 mM NaCl, and 0.1% Na3VO4 containing 2% bovine serum albumin to block unspecific binding. Preclarred supernatants 10 µl incubated overnight with anti-LIMK2 antibody (60 dilution) followed by the addition of 50 µl of 50% protein A-Sepharose slurry and incubation at 4 °C for 1 h. The immunoprecipitates were collected by centrifugation at 16,000 x g for 25 s then washed three times with 1 ml of ice-cold 1 x immunoprecipitation lysis buffer. The immuno-precipitates were further processed for immunoblot analysis.

**In Vitro Phosphorylation of LIMK2 by PKC**—EGFP-LIMK2 and mutant forms of EGFP-LIMK2 were immunoprecipitated from transfected endothelial cells. The immunoprecipitates were washed twice with PKC kinase buffer (20 mM HEPEs (pH 7.4), 10 mM MgCl2, 100 µM CaCl2). LIMK2 immunoprecipitates or recombinant His-LIMK2 (0.5 µg) were incubated with 0.3 units of different PKC isoforms/ml at 30 °C in 100 µl of buffer containing 100 µM ATP, 0.03% Triton X-100, 100 µM of phosphatidylserine/ml, and 20 µg of dicycloglycerol/ml. After incubation, reactions were terminated by the addition of SDS-PAGE sample buffer and boiling the mixtures for 5 min. The phosphorylation of LIMK2 was analyzed after immunoblotting with anti-philospho-Ser PKC substrate antibody.

**Western Blot Analysis**—Unstimulated and stimulated endothelial cells or immunoprecipitate samples were dissolved in an equal volume of 2 x Laemmli buffer. Equal amounts of proteins in the samples were then mixed with SuperSignal West Pico chemiluminescent substrate (Pierce) and exposed to Hyperfilm (Amersham Biosciences). The films were scanned into TIF format using a ScanJet 5300C (Hewlett-Packard Company, Palo Alto, CA). Densitometric analysis of the proteins was done using the NIH Image and Photoshop (Adobe Systems) software. The optical density scale of the scanner was done by using the Kodak step tablet (optical density 0.0–3.0) as a reference image. The densitometric values of phosphorylated proteins were divided by the corresponding values of unphosphorylated proteins, respectively. Absorption of proteins in unstimulated control samples was set to 100%. Data are presented as mean ± S.E. of three independent experiments.

**Cell Cycle Analysis of Endothelial Cells**—Endothelial cells were grown on 2% agarose in G0/G1 phase by serum starvation (endothelial cell basal medium containing 0.4% serum) for 48 h. The h-value refers to the percentage of cells in S phase at this time point. The cells (0.1 x 10^6cells/well) were plated into a 6-well plate in endothelial cell complete medium (Promocell) or endothelial basal medium containing 5% serum or 200 nM PMA. The cells were harvested at various time points and fixed in ice-cold methanol at 4 °C for 30 min. The cells were washed twice in 500 µl of phosphate-buffered saline containing 1% FCS, and the final pellet was resuspended in buffer (20 µg/ml propidium iodide, 10 µg/ml RNase, 0.1% Triton-X-100 in phosphate-buffered saline) and incubated for 1 h. The cells were analyzed using a FACScan flow cytometer. Data were analyzed using the cellQuest® software (BD Biosciences, San Jose, CA).

**Confocal Microscopy**—After 24 h of transfection, cells were transfected into phenol red-free OPTI-MEM® I medium (Invitrogen) and kept for 1–2 h in an incubator. Unstimulated and PMA-stimulated cells were observed with a Zeiss LSM510 confocal laser-scanning microscope. The cells were kept under the microscope at 37 °C. The 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.
PKC Inhibits Nuclear Import of LIMK2

Z-stacking, the top and the bottom positions 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 by LSM 510 software. The measurements were carried out in three independent experiments with 20 cells randomly selected in each experiment. Mean ± S.E. was calculated for each experiment.

FRAP and FLIP Analysis—Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) analysis was performed with the Zeiss LSM 510 confocal laser-scanning microscope as described previously (22). Briefly, an argon laser with a maximum output of 25 milliwatts (1 mW = 1 miliwatt) was used at 100% laser transmission with 20–40 iterations. For FRAP analysis, the entire nucleus was photobleached. The recovery of fluorescence intensity was measured in every 30 s in 12-bit fashion (4,096 gray tones). The average of the area of measurement and intensity was calculated, and the highest value of the four initial images before photobleaching was set to 100% intensity.

For FLIP experiments, the settings for image capture were the same as for the FRAP experiments, and images were recorded every 30 s. An area of cytoplasm was repeatedly bleached with 40 iterations at 100% laser transmission. The relative fluorescence in the nucleus of the bleached cell and an adjacent cell (unbleached control) was then measured.

RESULTS

LIMK2 Shuttles between the Cytoplasm and the Nucleus—To investigate the intracellular distribution of LIMK2 in endothelial cells, cells were transiently transfected with an expression vector encoding full-length LIMK2 tagged with EGFP. As control, the empty vector EGFP-C1 encoding EGFP protein was transfected into the cells. The EGFP protein was diffusely distributed throughout the cytoplasm and the nucleus (Fig. 1A, panel a), reflecting the unfacilitated diffusion of EGFP (~27 kDa) through the nuclear pore. The EGFP-LIMK2 protein was mainly localized in the cytoplasm (Fig. 1A, panel b), and only a small fraction was in the nucleus (Fig. 1B).

To determine whether LIMK2 shuttles between the nucleus and cytoplasm in endothelial cells, EGFP-LIMK2-transfected endothelial cells were treated with LMB (10 ng/ml), which inhibits the CRM1-dependent export of proteins from the nucleus (23, 24). After LMB treatment, EGFP-LIMK2 localized exclusively in the nucleus (Fig. 1A, panel d). The cytoplasmic distribution of EGFP was not affected by the LMB treatment (Fig. 1A, panel c). These results indicated that the cytoplasmic EGFP-LIMK2 continuously enters the nucleus and that CRM1 transports EGFP-LIMK2 actively and continuously out of the nucleus.

The nucleocytoplasmic shuttling of LIMK2 was directly visualized by photobleaching techniques in living endothelial cells. Endothelial cells were transfected with the EGFP-PDZ kinase domain construct of LIMK2 (EGFP-PDZK), which distributes equally between the nucleus and cytoplasm (Fig. 2A). Analysis by FRAP and FLIP showed that EGFP-PDZK shuttled between nucleus and cytoplasm. Specifically, FRAP analysis after photobleaching of the nucleus showed that EGFP-PDZK entered the nucleus. The time for 50% recovery ($\tau_o$) of fluorescence was 205 s (supplementary Fig. 1). These results suggested that the NLSs within the kinase domain drive the EGFP-PDZK into the nucleus.

To study whether EGFP-PDZK was exported from the nucleus to the cytoplasm, the FLIP analysis was used. An area of the cytoplasm of a cell expressing EGFP-PDZK was photobleached, and fluorescence intensity in the nucleus of the photobleached cell was monitored. As control, the fluorescence intensity in the nucleus of an adjacent unbleached cell was measured. The level of nuclear fluorescence in the photobleached cell gradually decreased over time. In contrast, the nuclear fluorescence in the adjacent cell (unbleached) was unaffected (supplementary Fig. 2). This indicated that EGFP-PDZK was exported from the nucleus in endothelial cells.

LIM Domains Regulate the Cytoplasmic Localization of LIMK2, Not LIMK1—Previously, it has been reported that LIM domains interact with the kinase domain of LIMK1 (25). Since LIMK2 is found mainly in the cytosol of resting endothelial cells, the putative NLS of LIMK2 in the kinase domain might be masked by the interaction with the LIM domains. To investigate this, different constructs of LIMK2 were made with EGFP in which either one or both LIM domains were deleted. The nuclear localization of ΔLIM1-LIMK2 (LIM1 domain deleted) was ~2 times higher than the wild type EGFP-LIMK2 (Fig. 2, A and B). This indicated that LIM1 plays a role in the cytoplasmic localization of LIMK2. In contrast, the ΔLIM2-LIMK2 protein (LIM2 domain deleted) was localized mainly in the cytoplasm, similarly to the full-length EGFP-LIMK2 protein, suggesting that the LIM2 domain alone does not affect the subcellular localization of LIMK2. EGFP-PDZK of LIMK2 (both LIM domains deleted) was drastically increased in the nucleus (to 40% of the total protein; Fig. 2B). These results suggested that both LIM domains contribute to the cytoplasmic localization of LIMK2. Also, EGFP-LIMK1 was mainly present...
PKC Inhibits Nuclear Import of LIMK2

The LIM domains of LIMK2 inhibit nuclear localization of LIMK2 but not LIMK1. Endothelial cells were transfected with various EGFP constructs of LIMK2 or LIMK1, and cellular localization was examined by confocal microscopy 24 h after transfection. A, representative fluorescence micrographs. Panel a, deletion of LIM1 domain (EGFP-ΔLIM1-LIMK2, amino acids 1–62 deleted) of LIMK2 enhanced the nuclear localization of EGFP-LIMK2. Panel b, the deletion of LIM2 domain (EGFP-ΔLIM2-LIMK2, amino acids 72–124 deleted) did not affect the cytoplasmic localization of LIMK2. Panel c, deletion of both LIM1 and LIM2 domains (EGFP-PDZK, amino acids 1–141 deleted) of LIMK2 drastically increased (40%) its localization in the nucleus. Panel d, in contrast, deletion of both LIM1 and LIM2 domains (EGFP-PDZK, amino acids 146–647) of LIMK1 did not affect the cytosolic localization of LIMK1. B, Bar diagram showing the nuclear fluorescence (percentages of total mean fluorescence intensity of the cell) of the different EGFP constructs of LIMK2 or LIMK1. Values are mean ± S.E. of three independent experiments. The asterisks denote statistical significance with respect to EGFP-LIMK2; *, p < 0.05; **, p < 0.01.

in the cytoplasm, and after LMB treatment, it was exclusively localized in the nucleus of endothelial cells (data not shown), COS cells, and Hela cells (27). However, in contrast to LIMK2, deletion of the two LIM domains of LIMK1 did not affect the subcellular distribution of LIMK1. The EGFP-PDZK of LIMK1 was still localized in the cytoplasm (Fig. 2, A and B). This indicated that the nuceloctytoplasmic shuttling of LIMK1 and LIMK2 is regulated by different mechanisms.

LIM domains might either inhibit the nuclear import or accelerate the nuclear export of LIMK2. To distinguish between these two mechanisms, the kinetics of nuclear accumulation of EGFP-LIMK2- and EGFP-PDZK-LIMK2-transfected endothelial cells were compared after the addition of LMB to inhibit nuclear export. We found that the nuclear fluorescence of EGFP-PDZK-LIMK2 increased twice as rapidly (from 40 ± 6 to 95 ± 4%; mean ± S.E.) as the nuclear fluorescence of EGFP-LIMK2 (from 6 ± 2 to 35 ± 7%; mean ± S.E.) 20 min after LMB addition. These results indicated that the LIM domains reduced the rate of the nuclear import of LIMK2; they were, however, unable to inhibit nuclear accumulation of LIMK2 when cells were treated for 1 h with LMB (Fig. 1A, panel d).

Effect of PMA on the Subcellular Localization of LIMK2—Analysis of the amino acid sequence revealed two potential PKC phosphorylation sites in LIMK2; one site at Ser-283 is present in the kinase domain, and the predicted NES is present C-terminal of the PDZ domain. A, a schematic representation of LIMK2 and their domains with the putative PKC phosphorylation site at Ser-283. The putative NLS is present in the kinase domain, and the predicted NES is present C-terminal of the PDZ domain. B, representative fluorescence micrographs of EGFP constructs in control cells (panels a–d) and after PMA treatment of endothelial cells (200 nM, 30 min; panels e and f). Panels a and e, EGFP-LIMK2; panels b and f, EGFP-PDZK of LIMK2; panels c and g, EGFP-kinase of LIMK2; panels d and h, EGFP-PDZK of LIMK1. C, bar diagram showing the inhibition of nuclear localization of EGFP-constructs of LIMK2 by PKC activation and the effect of two different PKC inhibitors. Endothelial cells were incubated with the PKC inhibitor Go6976 (2 μM) or Go6983 (500 nM) for 30 min and then stimulated with PMA for 30 min. Values are mean ± S.E. of three independent experiments. The asterisks denote statistical significance (*, p < 0.05; **, p < 0.01) with respect to cells not treated with PMA.

To explore whether PKC activation affects the nuclear localization of LIMK2, the subcellular distribution of EGFP-LIMK2, EGFP-PDZK, and EGFP kinase of LIMK2 in endothelial cells was studied before and after stimulation with PMA, a potent PKC activator. Within 30 min of stimulation with PMA, the amount of nuclear EGFP-LIMK2 (6 ± 2%) decreased to barely detectable levels (1 ± 0.5%, p < 0.05; Fig. 3B, panels b and e, and 3C). Nuclear EGFP-PDZK of LIMK2 was shifted to the cytoplasm in PMA-treated cells (Fig. 3B, panels b and f, and
PKC inhibits nuclear import of LIMK2

There was no effect of PMA on the nuclear localization of EGFP kinase of LIMK2 (Fig. 3B, panels c and g). These results indicated that PKC activation inhibits the nuclear localization of LIMK2 and suggested that Ser-283 between the PDZ and kinase domain may be the target for PKC phosphorylation, whereas the second potential phosphorylation site, Thr-494 in the kinase domain, does not seem to be involved in the regulation of LIMK2 nucleocytoplasmic shuttling. The subcellular localization of EGFP-PDZK of LIMK1 was not affected by PMA stimulation, indicating that nucleocytoplasmic shuttling of LIMK1 is not regulated by PKC activation (Fig. 3B, panels d and h).

To confirm that the PMA-induced exclusion of LIMK2 from the nucleus is regulated by PKC, two specific PKC inhibitors were used, Go6983 and Go6976 (28–30). The PMA-stimulated translocation of EGFP-PDZK to the cytoplasm was completely blocked by these two PKC inhibitors (Fig. 3C).

**PKC-dependent Phosphorylation of LIMK2 at Ser-283 in PMA-stimulated Endothelial Cells**—Phospho-specific antibodies are powerful tools to analyze protein phosphorylation. To investigate the potential PKC phosphorylation site in LIMK2, a specific anti-phospho-Ser PKC substrate antibody was used (31). This antibody binds with high affinity to the consensus peptide sequence (XXRRRS*LRRXX) for PKC phosphorylation present between the PDZ and kinase domain of LIMK2 (Fig. 3A).

To analyze whether endogenous LIMK2 was phosphorylated by PKC, endothelial cells were untreated (control) or treated with PMA, and LIMK2 in endothelial cell lysates was immunoprecipitated (IP) with a specific anti-LIMK2 antibody. The immunoprecipitates were subjected to SDS-PAGE and blotted with anti-phospho-Ser PKC substrate antibody and anti-LIMK2 antibody. Bar diagram of the densitometric analysis of the immunoblots. Results are mean ± S.E. of three independent experiments. C, mutational analysis of the Ser-283 phosphorylation site in LIMK2. Endothelial cells were transfected with wild type EGFP-PDZK-LIMK2 and its mutant (S283A). After 24 h of transfection, cells were not treated or treated with 200 nM PMA for 30 min. EGFP-PDZK proteins were immunoprecipitated with anti-LIMK2 antibody and blotted with anti-phospho-Ser PKC substrate antibody. D, PKC-δ phosphorylates LIMK2 at Ser-283. Upper blot, in vitro kinase assays were performed with different PKC isoforms and recombinant LIMK2. Proteins were immunoblotted with anti-phospho-Ser PKC substrate antibody. Lower blot, in vitro kinase assays were performed with PKC-δ and LIMK2 immunoprecipitates of endothelial cells transfected with EGFP, EGFP-PDZK-LIMK2, and EGFP-PDZK-LIMK2 (S283A). The immunoprecipitates were blotted with anti-phospho-Ser PKC substrate antibody and anti-LIMK2 antibody.
PKC-δ Phosphorylates LIMK2 at Ser-283 in Vitro—To investigate whether PKC directly phosphorylates LIMK2 and to analyze which PKC isoform is involved, we performed in vitro kinase assays using recombinant LIMK2 and different isoforms of PKC. The result demonstrates that only PKC-δ was able to phosphorylate LIMK2 (Fig. 4D, upper blot). To confirm that PKC-δ phosphorylated LIMK2 at Ser-283, immunoprecipitates of transfected EGFP-LIMK2 and its mutant S283A were subjected to in vitro phosphorylation by PKC-δ. The results demonstrate that PKC-δ phosphorylated wild type EGFP-LIMK2 but failed to phosphorylate the S283A mutant of EGFP-LIMK2 (Fig. 4D, lower blot). Together, the results proved that PKC-δ phosphorylates LIMK2 at Ser-283.

PKC-mediated Phosphorylation of Ser-283, but Not Thr-494, Inhibits the Nuclear Translocation of LIMK2—To examine whether Ser-283 phosphorylation is responsible for the translocation of LIMK2 from the nucleus to the cytoplasm in PMA-activated cells, Ser-283 was modified to either alanine (S283A) or two glutamic acids (S283EE). Substitution of the phosphorylation site with two acidic amino acids (EE) mimics the phosphorylation of that site. LIMK2 constructs containing the S283EE mutation should be constitutively active, whereas constructs containing the S283A mutation should be inactive. After PMA stimulation of cells, wild type EGFP-PDZK was excluded from the nucleus, whereas the nuclear localization of the S283A mutant of EGFP-PDZK was not changed (Fig. 5, A and B). In contrast, mutation of the second potential PKC phosphorylation site had no effect; the T494A mutant of EGFP-PDZK-LIMK2 was translocated from the nucleus to the cytoplasm similar to the wild type protein after PMA treatment (Fig. 5, A and B). In cells transfected with the active S283EE mutants of EGFP-PDZK-LIMK2 or EGFP-LIMK2, the protein was exclusively localized in the cytoplasm similar to the PMA-stimulated cells (Figs. 5A and 6). These results indicated that PKC regulates the nuclear transport of LIMK2 in PMA-activated endothelial cells through phosphorylation of Ser-283.

Serum Stimulation of Endothelial Cells Inhibits the Nuclear Localization of PDZK-LIMK2 by Protein Kinase C-dependent Phosphorylation of Ser-283—To provide evidence for the physiological relevance of the results, endothelial cells were stimulated with serum, which activates growth stimulatory signaling pathways such as PKC. FCS (10%) stimulation of endothelial cells, which had been serum-starved before for 15 h, inhibited the nuclear accumulation of EGFP-PDZK-LIMK2 similar to PMA. This effect was abolished by pretreatment of cells with Go6983 or by S283A mutation of EGFP-PDZK (Fig. 5, A and C). These results showed that exposure of endothelial cells to physiological growth factors present in serum-induced exclusion of LIMK2 from the nucleus, which was mediated by PKC phosphorylation of LIMK2 at Ser-283.

PKC-mediated LIMK2 Phosphorylation of Ser-283 Inhibits Nuclear Import of LIMK2—The exclusion of LIMK2 from the nucleus by PKC activation may be due to acceleration of nuclear export or inhibition of nuclear import. To distinguish between these two mechanisms, EGFP-LIMK2-transfected endothelial cells were treated with PMA, and LMB was then added to inhibit nuclear export. The kinetics of nuclear fluorescence of EGFP-LIMK2 after LMB addition showed a drastic inhibition of nuclear accumulation of LIMK2 after PMA pretreatment. Similar results were obtained in cells transfected with the active S283EE mutants of EGFP-LIMK2 (Fig. 6). These results indicated that phosphorylation of Ser-283 by PKC inhibits the nuclear import of LIMK2.

Figure 5. Effect of Ser-283 mutation on the nuclear localization of PDZK-LIMK2. Serum inhibits nuclear localization of PDZK-LIMK2 by protein kinase C-dependent phosphorylation of Ser-283. Wild type, S283A, S283EE, or T494A mutant plasmids of EGFP-PDZK-LIMK2 were transfected into endothelial cells. After 24 h, cells were activated with PMA (200 nM, 30 min). For serum stimulation, cells were starved for 15 h. A, representative fluorescence micrographs. The inhibition of nuclear localization of EGFP-PDZK by PMA treatment was abolished by S283A mutation but not by T494A mutation. The active S283EE mutant of EGFP-PDZK-LIMK2 mimics the effect of PMA. Serum inhibits the nuclear localization of EGFP-PDZK in 50–60% of the cells, which was abolished by S283A mutation. B, bar diagram showing the effect of serum on the nuclear localization of EGFP-PDZK, the inhibition by S283A mutation, and the PKC inhibitor Go6983. Cells were serum-starved for 15 h and incubated for 30 min at 37 °C with or without the PKC inhibitor Go6983 (1 µM) before stimulation with FCS (10%) for 1 h. Values are mean ± S.E. of three independent experiments. C, bar diagram showing the effect of serum on the nuclear localization of EGFP-PDZK, the inhibition by S283A mutation, and the PKC inhibitor Go6983. Cells were serum-starved for 15 h and incubated for 30 min at 37 °C with or without the PKC inhibitor Go6983 (1 µM) before stimulation with FCS (10%) for 1 h. Values are mean ± S.E. of three independent experiments.
PKC Activation Stimulates Cyclin D1 Expression and S-phase Entry of Endothelial Cells—Since it has been reported that nuclear localization of LIMKs can mediate suppression of cyclin D1 expression and inhibition of G1 phase cell cycle progression (19), we asked whether protein kinase C activation affects cyclin D1 expression and G1-to-S-phase transition of endothelial cells. Stimulation of endothelial cells, which were arrested at the G0/G1 phase of the cell cycle by prolonged serum starvation with PMA or serum, showed an accelerated S-phase entry 16–24 h after treatment. Cyclin D1 expression known to stimulate G1 phase cell cycle progression and S-phase entry was enhanced 8 h after PMA treatment and reduced by the PKC inhibitor Go6983 (Fig. 7). These results showed that PKC activation enhances cyclin D1 expression and subsequent G1 phase progression of endothelial cells, probably through PKC-mediated exclusion of LIMK2 from the nucleus, and relieved suppression of cyclin D1 expression.

PMA Activation of Endothelial Cells Does Not Stimulate LIMK-mediated Phosphorylation of Cofilin—PKC-induced phosphorylation of Ser-283 of LIMK2 might affect the kinase activity of LIMK2 and/or guide the enzyme to cofilin. Endothelial cells were stimulated with PMA, and the phosphorylation of LIMKs at Thr-505/Thr-508 (reflecting LIMK activation) and LIMK-mediated phosphorylation of cofilin were measured. PMA in contrast to thrombin did not stimulate Thr-505/Thr-508 phosphorylation of LIMK and cofilin phosphorylation in endothelial cells (Fig. 8 and data not shown). Therefore the PKC-mediated phosphorylation of LIMK2 affects the nucleocytoplasmic shuttling of LIMK2 but not its kinase activity toward cofilin.

DISCUSSION

In this study, we demonstrated a role of PKC in the regulation of nucleocytoplasmic shuttling of LIMK2 in endothelial cells. PKC phosphorylates LIMK2 at Ser-283, thereby inhibiting the translocation of LIMK2 from the cytoplasm to the nucleus. Macromolecules larger than 40–60 kDa are, in most cases, actively transported across the nuclear pore complex. The active nuclear import and export of proteins are mediated by specific amino acid sequences, NLSs and NESs, respectively (32), which are also present in LIMK1 (27). Previously, it has been demonstrated that LIMK1 has two NES at the C terminus of the PDZ domain and one NLS in the kinase domain. After LMB treatment, LIMK1 was predominantly localized in the nucleus, indicating that it shuttles between the nucleus and the cytoplasm (19, 27). We observed that similar to LIMK1, LIMK2 was exclusively present in the nucleus of LMB-treated endothelial cells, indicating that LIMK2 also shuttles between the nucleus and the cytoplasm. The existence of functional NES and NLS in LIMK2 was further proved by FRAP and FLIP analysis of the EGFP-PDZ kinase domain of LIMK2 in endothelial cells. Although both LIMK1 and LIMK2 shuttle between the nu-
PKC Inhibits Nuclear Import of LIMK2

How does PKC-mediated phosphorylation of LIMK2 regulate its nuclear cytoplasmic shuttling? Many nuclear cytoplasmic shuttling proteins such as diacylglycerol kinase \( \zeta \), Ca\(^{2+}\)/calmodulin-dependent protein kinase II, and cyclin B1 are phosphorylated near their NLS, thereby affecting their affinity to the importin protein complex (38–40). Ser-283 is far away from the predicted NLS in the primary sequence of LIMK2, indicating that Ser-283 phosphorylation is not directly affecting the binding of NLS of LIMK2 to importin-\( \alpha \). LIMK2 phosphorylated at Ser-283 might bind proteins such as 14-3-3, masking the NLS. This protein has been shown to bind to nuclear cytoplasmic shuttling proteins after their phosphorylation and to mask their NLS, thereby keeping these proteins in the cytoplasm (41, 42).

Acknowledgments—The technical assistance of Nicole Wilke is greatly appreciated. We thank Dr. Ralph Graf and Prof. Michael Schleicher for introduction and access to confocal microscope. We are grateful to Sadhna Goyal for help in preparation of the manuscript.

REFERENCES

1. Folkman, J., and Shing, Y. (1992) J. Biol. Chem. 267, 10931–10934
2. Jackson, D., Volpert, O. V., Beuck, N., and Linzer, D. I. (1994) Science 266, 1581–1584
3. Tang, S., Morgan, K. G., Parker, C., and Ware, J. A. (1997) J. Biol. Chem. 272, 27670–27671
4. van Nuen en Amerongen, G. P., Koolwig, P., Versteilen, A., and van Hinsberg, V. W. M. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 211–217
5. Arber, S., Barbayannis, F. A., Hasser, H., Schneider, C., Stanyon, C. A., Minden, A., and Bernard, O. (1998) Nature 393, 805–809
6. Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. (1998) Nature 393, 809–812
7. Amano, T., Kaji, N., Ohashi, K., and Mizuno, K. (2002) J. Biol. Chem. 277, 22093–22102
8. Sumi, T., Matsumoto, K., and Nakamura, T. (2002) Biochem. Biophys. Res. Commun. 290, 1315–1320
9. Ohashi, K., Nagata, K., Mekawa, M., Ishizaki, T., Narumiya, S., and Mizuno, K. (2000) J. Biol. Chem. 275, 3577–3582
10. Edwards, D. C., Sanders, L. C., Bokoch, G. M., and Gill, G. N. (1999) Nat. Cell Biol. 1, 253–259
11. Dan, C., Kelly, A., Bernard, O., and Minden, A. (2001) J. Biol. Chem. 276, 32115–32121
12. Sumi, T., Matsumoto, K., and Nakamura, T. (2001) J. Biol. Chem. 276, 670–676
13. Amano, T., Tanabe, K., Eto, T., Narumiya, S., and Mizuno, K. (2001) Biochem. J. 354, 149–159
14. Yang, N., Higuchi, O., and Mizuno, K. (1998) Exp. Cell Res. 241, 242–252
15. Nunoue, K., Ohashi, K., Okano, I., and Mizuno, K. (1995) Oncogene 11, 701–710
16. Irie, H., Ohashi, K., and Mizuno, K. (1998) Biochem. Biophys. Res. Commun. 246, 307–312
17. Osada, H., Hasada, K., Inazawa, J., Uchida, K., Ueda, R., Takahashi, T., and Nakamura, T. (2000) J. Biol. Chem. 277, 3577–3582
18. Osada, H., Hasada, K., Inazawa, J., Uchida, K., Ueda, R., Takahashi, T., and Nakamura, T. (1996) Biochem. Biophys. Res. Commun. 239, 582–589
19. Takahashi, H., Koshizumi, U., Miyazaki, J., and Nakamura, T. (2002) Dev. Biol. 241, 259–272
20. Roovers, K., Klein, E. A., Castagnino, P., and Assasin, R. K. (2003) Dev. Cell 5, 273–284
21. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) J. Clin. Invest. 52, 2745–2756
22. Ear, T., Giguet, P., Fleury, A., Starkova, J., Payet, M. D., and Dupuis, G. (2001) J. Immunol. Methods 257, 41–49
23. Ellenberg, J., and Lippincott-Schwartz, J. (1998) in Cells: A Laboratory Manual (Spector, D. L., Goldman, R. D., and Leinwand, L. A., eds) pp. 21.1.1–21.1.24, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanaigida, M., Horinouchi, S., and Yoshida, M. (1998) Exp. Cell Res. 242, 540–547
25. Wolff, B., Sugner, J. J., and Wang, Y. (1997) Chem. Biol. 4, 139–147
26. Nagata, K., Ohashi, K., Yang, N., and Mizuno, K. (1999) Biochem. J. 343, 99–105
27. Hiroaka, J., Okano, I., Higuchi, O., Yang, N., and Mizuno, K. (1996) FEBS Lett. 399, 117–121
28. Yang, N., and Mizuno, K. (1999) Biochem. J. 338, 793–798
29. Peterman, E. E., Taormina, P., II, Harvey, M., and Young, L. H. (2004) J. Cardiovasc. Pharmacol. 43, 645–656
30. Geschwindt, M., Dieterich, S., Rennecke, J., Kittlein, W., Mueller, H.-J., and Johannes, F.-J. (1996) FEBS Lett. 392, 77–80
31. Martiny-Baron, G., Kazanietz, M., Mischak, H., Blumberg, P., Koehs, G., Hug, M., Marce, D., and Schaad, C. (1993) J. Biol. Chem. 268, 9184–9197
32. Zhang, H., Zha, X., Tan, Y., Hornbeck, P. V., Mastrogianni, A. J., Alessi, D. R., Polakiewicz, R., and Comb, M. J. (2002) J. Biol. Chem. 277, 39379–39387
33. Gerlich, D., and Kutay, U. (1999) Annu. Rev. Cell Dev. Biol. 15, 697–706
34. Edwards, D. C., and Gill, G. N. (1999) J. Biol. Chem. 274, 11352–11361
35. Jiang, W., Kahn, S. M., Zhou, P., Zhang, Y. J., Cacace, A. M., Infante, A. S., Doi, S., Santella, R. M., and Weinstein, I. B. (1993) Oncogene 8, 3447–3457
36. Resnitsky, D., Gossen, M., Bujard, H., and Reed, S. (1994) Mol. Cell. Biol. 14, 1669–1679
37.Blobé, G. C., Obeid, L. M., and Hannun, Y. A. (1994) Cancer Metastasis Rev. 13, 419–434
37. Soh, J.-W., and Weinstein, I. B. (2003) J. Biol. Chem. 278, 34709–34716
38. Heist, E. K., Srinivasan, M., and Schalman, H. (1998) J. Biol. Chem. 273, 19763–19771
39. Topham, M. K., Bunting, M., Zimmerman, G. A., McIntyre, T. M., Blackshear, P. J., and Prescott, S. M. (1998) Nature 394, 697–700
40. Moore, J. D., Yang, J., Truant, R., and Kornbluth, S. (1999) J. Cell Biol. 144, 213–224
41. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) Cell 96, 857–868
42. Zhang, S., Xing, H., and Muslin, A. J. (1999) J. Biol. Chem. 274, 24865–24872