p57^{kip2} Regulates Actin Dynamics by Binding and Translocating LIM-kinase 1 to the Nucleus*

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Abstract

p57^{kip2} is the only cyclin-dependent kinase (Cdk) inhibitor shown to be essential for mouse embryogenesis. The fact suggests that p57 has a specific role that cannot be compensated by other Cdk inhibitors. LIM-kinase 1 (LIMK-1) is a downstream effector of the Rho family of GTPases that phosphorylates and inactivates an actin depolymerization factor, coflin, to induce the formation of actin fiber. Here we demonstrate that p57 regulates actin dynamics by binding and translocating LIMK-1 from the cytoplasm into the nucleus, which in turn results in a reorganization of actin fiber. The central region of p57, a unique feature among the Cdk inhibitors, and the N-terminal region of LIMK-1, which contains the LIM domains were essential for the interaction. Expression of p57, but not p27^{kip1} or a p57 mutant, with a deletion in the central region was shown to induce marked reorganization of actin filament and a translocation of LIMK-1. Our findings indicate that p57 may act as a key regulator in embryogenesis by bearing two distinct functions, the regulation of cell cycle through binding to Cdns and the regulation of actin dynamics through binding to LIMK-1, both of which should be important in developmental procedure.

In mammals, cell cycle progression is governed by sequential formation, activation, and subsequent inactivation of a series of cyclin-cyclin-dependent kinase (Cdk) complexes. The activity of cyclin-Cdk is also regulated positively and negatively by phosphorylation (1). In these regulatory processes, cyclin-Cdk positively drives the progression of the cell cycle, whereas Cdk inhibitory protein (CKI) acts as negative regulators by binding to cyclin-Cdk complexes. On the basis of homology and specificities of interactions, CKIs can be classified into two distinct families, i.e. the INK4 (inhibitor of Cdkks) family and the Cip/kip (Cdk-interacting protein/kinase inhibitory protein) family. The INK4 family (p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, and p19^{INK4D} (2–6)) inhibits only Cdk4 and Cdk6, whereas the Cip/Kip family (p21^{CIP1}, p27^{kip1}, and p57^{kip2} (7–15)) inhibits multiple Cdns including both Cdk4/6 and Cdk2 by binding to cyclin-Cdk complexes.

The amounts of CKIs expressed in cells may be crucial in controlling cells regarding whether to start or to stop proliferating. The Cip/Kip family of CKIs is known to induce cell cycle arrest in response to antiproliferative stimuli, including contact inhibition, serum deprivation, and differentiation in vitro (1). Conversely, the p27 level was shown to be decreased following mitogenic stimulation of quiescent cells in a number of systems (16–19) where the ubiquitin-proteasome pathway is known to play an essential role (17). There are convincing lines of evidence addressing the importance of the Cip/Kip family proteins in the proliferation and/or differentiation of cells. Their expression patterns during development, where accumulation has been shown in many terminally differentiated cells (1), implicate them as the primary effectors that control cell cycle exit, which is critical for differentiation (20–24). Also, p21 and p27 are both shown to be induced during the differentiation process of certain cell types including muscle (20), neuronal (21, 22), and hematopoietic cells (23, 24). In some cases introduction of p21 or p27 into cognate precursor cells have been shown to induce the cells to differentiation. Therefore, the Cip/Kip family may have important roles in the withdrawal of the cells from the cell cycle in the differentiation process.

However, among the studies of mice deficient in CKIs, only p57 is shown to be essential for mouse embryogenesis, where neither the lack of p21, p27, nor INK family proteins show gross developmental defects (25, 26). These observations suggested that p57 might have a specific role in developmental process that cannot be compensated by other CKIs.

Developmental defects shown in p57 knockout mice include a cleft palate and abnormal endochondral ossification, attributable to increased apoptosis or proliferation at the expense of cell differentiation. Thus, it is of interest to explore the relationship between p57 expression and cell proliferation under physiological circumstances.

Recently, we found that p57 accumulates in primary cultured rat osteoblastic cells induced to differentiate by serum starvation. Furthermore, transforming growth factor β1 (TGFB1) was shown to re-stimulate the proliferation where reversing the differentiation process of osteoblastic cells by rapidly degrading p57 through proteasome-dependent pathway (27, 28). There was no significant change in the expressions of p21 and p27 in these experiments, which strongly suggested that...
p57 may play a specific role in the regulation of osteoblastic cell differentiation.

In the present study, to further investigate the significance of p57, we performed yeast two-hybrid screen to search for proteins interacting with p57 in primary cultured osteoblastic cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sources of materials were as follows: recombinant human TGF-β1 (R & D Systems Inc.), mouse monoclonal anti-HA antibody (Roche Diagnostics Co.), mouse monoclonal anti-c-Myc antibody (Roche Diagnostics Co.), mouse monoclonal anti-FLAG M2 antibody (Sigma), rabbit polyclonal anti-LIMK-1 antibody (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-p21CIP1 antibody (Santa Cruz Biotechnology, Inc.). Polyclonal antibodies against mouse p27Kip1 and p57Kip2 were raised in rabbits using synthetic peptides corresponding to the C-terminal amino acids of each protein. All antibodies cross-react efficiently with the respective mouse homologs.

**Isolation and Culture of Osteoblastic Cells**—Primary mouse osteoblastic cells were isolated from calvariae of 1-day-old mice, strain ddY, by sequential enzymatic digestion as described previously (27, 28). The cells were maintained in minimum essential medium α medium supplemented with 10% fetal bovine serum and antibiotics-antimycotics. Cells at the second passage were stocked and used for each experiment.

Induction of differentiation and TGF-β1 stimulation was performed as described previously (27, 28).

**cDNA Library**—mRNA was prepared from osteoblastic cells induced to differentiation by serum starvation, and cDNA was synthesized using random hexamers as the primer. The cDNA fragments were ligated into pG4-5 vector. The resulting library contained 6.5 × 108 independent clones with a cDNA insert size of 0.3–3 kb.

**Yeast Two-hybrid Screening**—The DupLEX-A yeast two-hybrid system (OriGene Technologies, Inc.) was used for the yeast two-hybrid screen. As for the bait constructs, the C-terminal fragments of the mouse p57 (p57C1, 267–335 amino acids; p57C2, 79–335 amino acids) were inserted into pEG202 vector, as a fusion to LexA DNA-binding domain. Screening was performed according to the manufacturer’s instructions. The clones interacted with p57C2 were subjected to DNA sequencing analysis by the dye terminator cycle sequencing method.

**Constructions**—The mouse LIMK-1 cDNA was obtained by reverse transcriptase polymerase chain reaction (PCR) method. Expression plasmids for full-length LIMK-1 and LIMK-1–159 were constructed by ligating each cDNA fragments into pcDNA3.1 (+) M vector (Invitrogen) using the PCR method. Expression plasmids for HA-tagged CKIs (pHA-p21, pHA-p27, pHA-p57 and pHA-p57Δ) were constructed by adding a HA-tag to each cdNA in pcDNA3.1(+) (Invitrogen). Expression plasmid for FLAG-tagged full-length mouse LIMK-1 (pFLAG-LIMK-1) was constructed by ligating the cDNA fragment into pCAGGS-LIMK-1 vector (Sigma). An expression plasmid for LIMK-1 with a dominant negative mutation (pFLAG-LIMK-1-D460A) was constructed using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

**Cell Culture, Transfection, Immunoprecipitation, and Immunoblot Analysis**—Human embryonic kidney (HEK) 293T and COS-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum. For transfection, 5 × 105 cells were grown in 100-mm culture dishes and were used for transfection, and cells were harvested or analyzed after 48 h. Immunoprecipitation and immunoblots were performed as described previously (27, 28).

**In Vitro Kinase Assays**—Immunoprecipitations of FLAG-tagged LIMK-1 with anti-FLAG antibodies were performed as described above. The immunoprecipitates were then washed three times with kinase assay buffer (50 mM Hepes (pH 7.2), 150 mM NaCl, 5 mM MgCl2, 10 mM NaF, 1 mM Na3VO4, 10 mM β-glycerophosphate, and 1 mM dithiothreitol) and mixed with 40 μl of kinase buffer containing 90 μM ATP, 5 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham Biosciences), and 2 μg of coflin (CytoSkelton, Inc.). The samples were incubated at 30 °C for 45 min, and the reactions were terminated by the addition of SDS sample buffer. The samples were separated on 18% SDS-PAGE. 32P-Labeled proteins were visualized by autoradiography.

**Immunocytochemistry**—For immunocytochemistry, cells were plated on coverslips in chambered slide glass (Falcon). Cells were fixed and permeabilized with a 4% formaldehyde, 0.1% Triton X-100 in PBS for 5 min. After blocking with PBS containing 1% bovine serum albumin for 10 min, cells were incubated with either anti-FLAG M2 monoclonal antibody or anti-HA monoclonal antibody, for 1 h at room temperature. After washing three times with PBS, cells were incubated with a fluorescein-anti-mouse IgG antibody (Dako) for 1 h at room temperature. For F-actin staining, rhodamine-conjugated phalloidin (Molecular Probes, Inc.) was added in incubation with the secondary antibody. After washing three times with PBS, cells were photographed on a fluorescence microscopy (Leica Microsystems Inc.).

**RESULTS**

**Interaction of p57Kip2 with LIMK-1–159 by Using Yeast Two-hybrid System**—To determine the relationship to p57 and cell growth and differentiation of osteoblastic cells, we searched for proteins interacting with p57 in osteoblasts by using a yeast two-hybrid system. A p57 fragment devoid of the Cdk inhibitory domain (p57C2; residues 79–335) was used as a bait (Fig. 1A). A complementary DNA (cDNA) library was prepared from primary cultured mouse osteoblastic cells induced to differentiation and was screened as a prey. Sequence analysis of the positive clones revealed that one clone contained the N-terminal portion of LIM-kinase 1 (LIMK-1–159, contains residues 1–159; Fig. 1A). LIMK-1 is a serine/threonine kinase with structures composed of two LIM domains and a PDZ domain in the N-terminal half and a kinase domain in the C-terminal half (Fig. 1A) and has been shown to phosphorylate and inactivate an actin depolymerization factor (ADF), coflin (29–34), which results in stabilization and accumulation of assembled actin filaments in the cells (29, 30). The clone obtained from our screening, LIMK-1–159, spanned the two LIM domains. The LIM domain is a double zinc finger motif found in a variety of proteins, including homeodomain-containing transcription factors, cytoskeletal-component proteins, and other signaling molecules, that is thought to be involved in protein-protein inter-

![Fig. 1. Interaction of p57Kip2 and LIMK-1–159 by the yeast two-hybrid screening, A](image-url)
action. Thus, it is likely that the LIM domains are responsible for the interaction of LIMK-1 with p57.

p57 has a Cdk inhibitory domain in its N-terminal region, which is well conserved among the Cip/Kip family of the CKIs, and a region termed QT box, which shares similarity with p27 in its C-terminal region (14, 15). In addition, p57 has a unique central region, consisting of a proline-rich region followed by an acidic repeat region in mouse or a region containing proline-alanine repeats in human (14, 15), where its significance remained unclear. Therefore we next investigated which part of the p57 is required for the interaction with LIMK-1. LIMK-1–159 did not show interaction with a shorter C-terminal fragment of p57 (p57C1, residues 267–335; Fig. 1A), nor with a C-terminal fragment of p27 (p27C, residues 95–127) in the yeast two-hybrid system (Fig. 1B and data not shown). Our findings indicated that the central region of p57 has an essential role for the interaction with LIMK-1 and suggested that the interaction is specific to p57.

Interaction of p57Kip2 and LIMK-1—We tested the interaction between p57 and LIMK-1 in overexpressed cells. HA-tagged full-length p57 and c-Myc-tagged LIMK-1–159 were transfected in the HEK 293T cells. Co-immunoprecipitation experiments confirmed the interaction of LIMK-1–159 with full-length p57 (Fig. 2A). Full-length LIMK-1 was also shown to interact with p57, but not with p27 or p21 in similar experiments, indicating that LIMK-1 interacts specifically to p57 (Fig. 2, B and C). Finally, interaction of endogenous p57 with LIMK-1 was also demonstrated in osteoblastic cells induced to differentiation by serum starvation (Fig. 2D).

p57Kip2 Binding Does Not Inhibit the Kinase Activity of LIMK-1—Next, we examined whether the binding of p57 affects the kinase activity of LIMK-1. FLAG-tagged LIMK-1 was expressed in HEK 293T cells, together with either p57, p27, with deletion in its unique central region (p57Δ, Fig. 1A), or p27 (Fig. 3A). LIMK-1 was immunoprecipitated from total cell extracts prepared from each cells, and the kinase activity was measured using cofillin as a substrate. Cofilin-phosphorylating activity of LIMK-1 was reduced in cells transfected with p57 compared with the cells transfected with p57Δ or p27 (Fig. 3A, upper panel). However, the amount of LIMK-1 expressed in cells transfected with p57 was also reduced compared with the cells transfected with p57Δ or p27 (Fig. 3A, lower panel). Relative kinase activities to phosphorylate cofillin did not differ significantly when they were normalized with the amounts of LIMK-1 expressed in each cell (Fig. 3B). Thus, we conclude that binding of p57 to LIMK-1 does not directly inhibit the kinase activity of LIMK-1. It is possible that the interaction may affect the expression level of LIMK-1, since reduced expression of LIMK-1 in cells co-transfected with p57 compared with the cells co-transfected with p57Δ or p27 was seen in repeated experiments, where the mechanism is not clear.

Effect of p57Kip2 on Actin Dynamics—Finally, we investigated whether this interaction affects the actin cytoskeleton of

**Fig. 2. Interaction of p57Kip2 and LIMK-1.** A, co-transfection and co-immunoprecipitation assay confirmed the interaction of p57 and the c-Myc-tagged LIMK-1–159 fragment in HEK293T cells. B, p57 bound to full-length LIMK-1 in HEK293T cells. C, LIMK-1 binds specifically to p57, but not with p21 or p27. Lane 1, HEK293T cells were transfected with FLAG-tagged LIMK1 expression plasmid (pFLAG-LIMK-1) as a control. Lanes 2 and 3, cells were co-transfected with HA-tagged p21 expression plasmid (pHA-p21) together with pFLAG-LIMK-1. Lanes 4 and 5, with HA-tagged p27 expression plasmid (pHA-p27) together with pFLAG-LIMK-1. Extracts obtained from each cells were immunoprecipitated with the antibodies indicated and blotted with anti-FLAG antibody to visualize LIMK-1, where no antibody was added in the negative controls (lanes 2, 4, and 6). The amounts of each CKIs immunoprecipitated in the experiment were confirmed by blotting the same membrane with anti-HA antibody (lower panel). D, binding of endogenous p57 with LIMK-1 was demonstrated in primary cultured osteoblastic cells induced to differentiation by serum starvation, where the binding was not seen when the cells were re-stimulated by TGF-β1.

**Fig. 3. p57Kip2 binding does not inhibit the kinase activity of LIMK-1.** A, HEK293T cells were co-transfected with pFLAG-LIMK-1 (lanes 1, 3, 4, and 5), or pFLAG-LIMK-1-D460A, a dominant negative mutant (lane 2), together with pcDNA (lanes 1 and 2), pHA-p57 (lane 3), pHA-p57Δ (lane 4), or pHA-p27 (lane 5). The lysates were immunoprecipitated with anti-FLAG antibody and subjected to kinase reaction in vitro. The kinase activity was measured by 32P incorporation into cofillin (upper panel). The middle panel indicates Coomassie Brilliant Blue (CBB) staining of cofillin. The amounts of the LIMK-1 protein immunoprecipitated and subjected in each assay was quantified by immunoblot analysis with anti-LIMK-1 antibody (lower panel). Expression of FLAG tagged LIMK-1 protein in the cells were quantified by immunoblot analysis with anti-FLAG antibody. B, activities to phosphorylate cofillin were normalized by the amount of LIMK-1 subjected in each reaction. LIMK-1 expressed in each cell (Fig. 3B). Thus, we conclude that binding of p57 to LIMK-1 does not directly inhibit the kinase activity of LIMK-1. It is possible that the interaction may affect the expression level of LIMK-1, since reduced expression of LIMK-1 in cells co-transfected with p57 compared with the cells co-transfected with p57Δ or p27 was seen in repeated experiments, where the mechanism is not clear.

Effect of p57Kip2 on Actin Dynamics—Finally, we investigated whether this interaction affects the actin cytoskeleton of
the cells. Forced expression of FLAG-tagged LIMK-1 in COS-7 cells induced the formation of thick bundled actin fibers, which is consistent with previous reports (29, 30). Co-expression of p57, but not p57Δ or p27, resulted in the marked dissolution of these fibers, which may reflect an inhibition of the activity of LIMK-1 (Fig. 4). This was intriguing, as p57 did not show significant inhibition of the kinase activity of LIMK-1 in our experiment (see above), p57, as a Cdk inhibitor, has been shown to be localized specifically in the nucleus. Since the localization of LIMK-1 has been reported to be cytoplasmic, thereby affecting the actin dynamics in the cytoplasm (35), we were prompted to investigate if the expression of p57 may affect the subcellular location of LIMK-1. As expected, in the cells co-expressing p57, LIMK-1 staining was demonstrated mainly in the nucleus, in sharp contrast with the cells expressing LIMK-1 alone, or cells co-expressing either p57Δ or p27, where LIMK-1 remained in the cytoplasm leaving the nucleus unstained (Fig. 4). p57, p57Δ, and p27 were all shown to be localized in the nucleus in duplicated experiments (data not shown).

DISCUSSION

In this paper, we have shown evidences that p57 binds to LIMK-1, both in vitro and in vivo, and can regulate actin dynamics through this interaction. LIMK-1 is a LIM domain containing protein kinase, which regulates actin dynamics through phosphorylation of an ADF family protein, cofilin at Ser-3. Actin depolymerization activity of cofilin is inactivated by this phosphorylation, which results in stabilization and accumulation of assembled actin filaments in the cells (29–34). LIMK-1 has been shown to be involved in the signaling pathway of Rho family of GTPases, Cdc42, Rac, and Rho, which is essential for the development and survival of various multicellular organisms (30, 31, 36). Recent studies have revealed that LIMK-1 can be directly activated through phosphorylation by Rho-associated protein kinases, ROCK and PAK (32, 33). Co-expression of p57 with LIMK-1 resulted in a translocation of LIMK-1 from the cytoplasm to the nucleus and a marked reorganization of actin fiber induced by the expression of LIMK-1. These effects were not seen in the cells co-expressing p27 or a mutant p57 lacking the region essential for binding to LIMK-1. Therefore, it was strongly suggested that the binding of p57 to LIMK-1 is essential for this regulation and is independent from the cell cycle arrest induced by the Cdk inhibitory activity of p57. As p57 is located exclusively in the nucleus in our experiment, it is likely that LIMK-1 was anchored in the nucleus by binding to p57. In addition, binding of p57 did not show direct effect on the kinase activity of LIMK-1, suggesting that p57 affects actin dynamics mainly by changing the location of LIMK-1 in the cells.

Based on our results, we propose the following model (Fig. 5). When excessive amount of p57 is expressed in the cells, p57 will bind LIMK-1 and translocates the kinase from cytoplasm into the nucleus, resulting in the depletion of LIMK-1 activity to phosphorylate and inactivate cofilin in the cytoplasm, which in turn results in induction of depolymerization of actin filament. Therefore, p57 may regulate actin dynamics simultaneously with the control of cell cycle. We should point out that it is also possible that LIMK-1 may have an additional role in the nucleus, as p57 did not affect the activity of LIMK-1 significantly in our experiment. Thus it will be important to elucidate the possible effect of the kinase in the nucleus.

p57 was shown to bind to the N-terminal region of LIMK-1, which contains two tandem repeat of a LIM motif. The LIM motif is composed of two adjacent putative zinc fingers, which have been suggested to be involved in protein-protein interactions and are found in diverse proteins known to be involved in cell fate determination, growth regulation, and oncogenesis, including homeodomain-containing transcriptional factors, cytoskeletal proteins, and other signaling proteins (37, 38). The LIM domains of LIMK-1 may be important for the regulation of actin dynamics.
cell differentiation. Neuronal differentiation of PC12 cells was inhibited by an expression of LIMK-1, where the LIM domain was shown to be essential for the inhibition (39, 40), suggesting an existence of LIM domain interacting proteins, which may play important roles in the regulation of cell differentiation.

Neuregulins, a family of transmembrane proteins that function as receptor tyrosine kinase ligands known to be involved in the regulation of synapse formation, have been shown to be a candidate of such proteins (41). Also, studies of several LIM-containing proteins, including focal adhesion plaque proteins, paxillin, zyxin, and cysteine-rich protein, have suggested that LIM domains are involved in their binding to other cytoskeletal regulatory or component proteins (38, 42–45). Therefore, it is important to further investigate if p57Kip2 blocks the binding of such protein to LIM domain, which may be important in the regulation of actin dynamics, cytoplasmic anchoring of LIMK-1, and cell differentiation.

Our data indicated that the central region of p57, a unique feature of p57 among the Cip/Kip family of CKIs, is essential for the binding and regulation of LIMK-1, where p27 was not involved in this regulation. This may in part explain why only p57, among the CKIs, is essential for embryogenesis. Embryonic development of a multicellular organism requires complex combination of cellular activities including control of cell proliferation, movement, deformation, and differentiation. p57 may be more important in this delicate regulation, not only by regulating cell cycle, but also by establishing a cross-talk between the regulation of cell cycle and the regulation of actin dynamics.

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