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Nischarin Inhibits LIM Kinase To Regulate Cofilin Phosphorylation and Cell Invasion▽

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Nischarin is a novel protein that regulates cell migration by inhibiting p21-activated kinase (PAK). LIM kinase (LIMK) is a downstream effector of PAK, and it is known to play an important role in cell invasion. Here we show that nischarin also associates with LIMK to inhibit LIMK activation, cofilin phosphorylation, and LIMK-mediated invasion of breast cancer cells, suggesting that nischarin regulates cell invasion by negative modulation of the LIMK/cofilin pathway. The amino terminus of nischarin binds to the PDZ and kinase domains of LIMK. Although LIMK activation enhances the interaction with nischarin, only phosphorylation of threonine 508 of LIMK is crucial for the interaction. Inhibition of endogenous nischarin expression by RNA interference stimulates breast cancer cell invasion. Also, nischarin small interfering RNA (siRNA) enhances cofilin phosphorylation. In addition, knock-down of nischarin showed branched projection actin structures. Collectively these data indicate that nischarin siRNA may enhance random migration, resulting in stimulation of invasion.

Tumor cell migration/invasion is an important factor in solid tumor formation and is necessary for the spread to different organs (47), and thus cellular invasion is a hallmark of metastasis (25). Cellular invasion is a complicated process that involves cytoskeletal reorganization, formation of lamellipodia, membrane ruffling, and altered cell morphology (47). For instance, cell invasion requires partial detachment from intercellular adhesions and from cell-extracellular matrix interactions, reorganization of the actin cytoskeleton, and movement through the extracellular matrix (47, 57). Thus, the actin cytoskeleton is an important determinant of cell motility and cell invasion (5). The actin cytoskeleton drives formation and extension of lamellae at the leading edge of the cells, while the actin-based molecular motor myosin provides the force necessary for cell movement (36).

Members of Rho family GTPases are crucial regulators of several biological events, and they are particularly important in the organization of the actin cytoskeleton, as well as in cell migration and invasion (26, 27, 29, 58). Several effectors of Rho GTPases have been identified, but signal transduction pathways that link these to the actin cytoskeleton are not completely understood. A number of actin-associated proteins that regulate actin polymerization and depolymerization are potential downstream mediators. For example, the biological effects of Rac are exerted through the activation of several downstream effectors (11). One important family of Rac effectors is the p21-activated kinases (PAKs), which play a role in cytoskeletal reorganization (9) and cell migration (30, 35). Actin cytoskeletal reorganization is initiated when PAK1 is activated by GTP-bound Rac or Cdc42. PAK then transphosphorylates and activates LIM kinase 1 (LIMK1). Active LIMK1 in turn catalyzes phosphorylation of an N-terminal serine residue of cofilin, thereby inactivating its F actin-depolymerizing activity and leading to accumulation of actin filaments and aggregates (7, 59). LIMK1 is overexpressed in prostate tumors and in prostate (16) and invasive breast cancer (61) cell lines. Overexpression of LIMK1 in MCF-7 and in MDA-MB-231 human breast cancer cell lines increases their motility, while inhibition of LIMK1 activity by expression of a dominant-negative LIMK1 resulted in decreased motility (61). Thus, LIMK1 may play a pivotal role in cancer cell invasion. LIMK regulates the G_2/M checkpoint and invasion (16). Further a chromosomal gain on 7q11.2 in metastatic prostate cancer has been shown to exist, which in turn may result in gain of LIMK (7q11.23), suggesting that LIMK is an oncogene (16, 17, 60). The ability of cancer cells to invade different parts of the body results in significant morbidity and mortality. Thus, understanding the mechanism of invasion and metastasis at the cellular and molecular levels could provide new diagnostic, prognostic, and therapeutic approaches to cancer (8, 16, 61).

In our previous studies, we identified a novel protein, nischarin, which binds selectively to the integrin α5 subunit cytoplasmic tail, inhibits cell motility, and alters actin filament organization (4). Nischarin inhibits Rac-induced cell migration and invasion in breast and colon epithelial cells (3) and also interacts with members of the PAK family to inhibit PAK1 activation (6). The amino terminus of nischarin binds to the kinase domain of PAK. Furthermore, we have shown that nischarin blocks cell invasion, affecting PAK signaling. Interestingly, nischarin also blocks PAK-independent Rac signaling by directly interacting with Rac (43). It is important to note that IRAS, the human ortholog of nischarin, binds to the adaptor protein IRS4, and the downstream effects are not clear yet (33). Also, IRAS has been shown to be colocalized to

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endosomes, and this colocalization depends upon the PHOX domain and coiled-coiled region in the amino terminus of IRAS. IRAS has been shown to play an important role in translocation of α5 integrin from the membrane to endosomes (33). Nischarin does not contain the PHOX domain; however, the coiled-coil region in the amino terminus of nischarin may be sufficient for regulation of translocation in mouse cells. Recent data indicate that nischarin plays an important role in neuronal signaling triggered by I1 receptor activation in rostral ventral medullary neurons (62). Thus, nischarin seems to have an important role in several biological processes.

Nischarin's ability to inhibit cell migration and invasion led us to hypothesize that nischarin may play a role in PAK1-LIMK1-regulated cell motility and invasion. Here we demonstrate that nischarin binds to LIMK1 and for the association, LIMK activity per se is not required but phosphorylation of threonine 508 of LIMK is essential. Nischarin blocks LIMK activation as well as cofilin phosphorylation. Furthermore, reduction of endogenous levels of nischarin stimulates cell invasion by affecting the actin cytoskeleton. This report demonstrates for the first time that nischarin is a negative regulator of LIMK1, and it identifies a novel signaling pathway in breast cancer cells by which nischarin-mediated regulation of LIMK1 and cofilin affects cell invasion.

MATERIALS AND METHODS

Reagents. Full-length mouse nischarin cDNA, all nischarin deletion mutants, and β-galactosidase (β-Gal) were expressed from pcDNA3.1 B Myc/His (Invitrogen, Carlsbad, CA). Nischarin was also expressed from the pEGFP-N1 vector (Clontech, Mountain View, CA). Hemagglutinin (HA)-tagged LIMK1, HA-LIMK2, Flag-LIMK1, Flag-LIMK1 PDZ, Flag-LIMK1 kinase domain, Flag LIMK1, -2, and the Flag LIMK kinase domain short were generous gifts from Ora Bernard. LIMK1 T508EE, LIMK1 T508V, and LIMK1 D460N were generous gifts from Gordon Gill. Full-length human PAK1 (6), T423E, K299R, and β-galactosidase were expressed from pcDNA3.1 B V5/His (Invitrogen, Carlsbad, CA). For the in vitro binding studies, glutathione *S*-transferase (GST)-tagged nischarin was expressed from the bacterial expression vector pGEX-4T-3 (Amersham Biosciences, Piscataway, NJ). Purified LIMK and cofilin were purchased from Invitrogen and Cytoskeleton (Denver, Co), respectively. LIMK and cofilin antibodies were purchased from Cell Signaling (Danvers, MA).

Cell culture. The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM; supplemented with nonessential amino acids; Invitrogen), 0.1 mM sodium pyruvate (Invitrogen), 10 μg/ml insulin (Invitrogen), 10% (vol/vol) fetal bovine serum (FBS; HyClone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cos-7 cells were cultured in DMEM with 10% FBS. Rat adrenal gland PC12 cells were maintained in DMEM with 10% FBS and 5% horse serum.

Immunoprecipitation and Western blotting. For nischarin/LIMK1 binding, Cos-7 cells were transfected with 2 μg each of nischarin and LIMK1 plasmids using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 1% NP-40, 0.1% sodium deoxycholate, 1 mM sodium pyrophosphate) with protease and phosphatase inhibitors. The lysates were incubated with anti-Myc antibody 9E10 (Covance, Berkeley, CA), anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-Flag antibody (Sigma, St. Louis, MO) at 4°C for 6 h. The immune complexes were precipitated with protein G-Sepharose and immunoblotted as described elsewhere (6). Briefly, the proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride membrane. All primary antibodies were used at a dilution of 1:1,000, horseradish peroxidase-conjugated secondary antibodies were used at a dilution of 1:5,000, and the horseradish peroxidase activity was detected with the ECL Western blotting system (GE Healthcare). X-ray films were scanned and imported into Adobe Photoshop, and the figures were prepared in Adobe Illustrator or Microsoft Power Point.

Phosphatase treatment. Cell lysates were prepared as described above in modified RIPA buffer using all protease inhibitors (phosphatase inhibitors were omitted), and the cell lysates were directly treated with 40 U of γ-phosphatase (New England Biolabs) for 1 hour at 37°C. We followed the procedure exactly as described by Treisman and colleagues (22).

Immunofluorescence. The detailed techniques for immunofluorescence have been described in our previous papers (4, 6). Briefly, MCF7 cells were transfected with nischarin small interfering RNA (siRNA) plus green fluorescent protein (GFP), GFP plus control siRNA, or GFP alone. After 24 h of transfection, cells were plated on fibronectin-coated coverslips for 3 hours in DMEM containing 10% FBS. Cells cultured on fibronectin-coated slides were fixed with 3.7% paraformaldehyde–phosphate-buffered saline followed by incubation with 0.5% Triton X-100 for 5 min at room temperature, washed with phosphate-buffered saline, blocked, and incubated with Alexa 546-conjugated phalloidin (Invitrogen) for 1 hour. Also, the cells cultured on fibronectin-coated coverslips were incubated with antitubulin (Sigma) for 1 h followed by incubation with Alexa 546-labeled (red) secondary antibody (Invitrogen). Slides were mounted in ProLong Gold Antifade (Invitrogen). Fluorescent images were acquired using a confocal scanning microscope (Leica DMIRE2) with a 63× 1.2 water immersion objective at room temperature.

Image processing and figure preparation. Images were exported into Adobe Photoshop. All submitted figures were either prepared in Microsoft Power Point or Photoshop.

Transwell cell invasion assay. Cell invasion assays were performed essentially as described elsewhere (3). Briefly, MCF7 cells were transiently transfected with constitutively active LIMK1 (HA-LIMK1) or with active PAK (V5-PAK-T423E) plus full-length Myc-nischarin. A pRC β-Gal plasmid (Stratagene, La Jolla, CA) was used as a marker. Also, sometimes GFP-nischarin was substituted for the Myc-nischarin plus β-Gal plasmid combination. The underside of the transwell was coated with fibronectin, and the upper surface was coated with 25% matrigel (BD Scientific, San Jose, CA). Cells were added to the upper surface. The β-galactosidase-positive cells that invaded through the membrane during a 48-h incubation were counted by staining for β-Gal. The ratio of invaded transfected cells to total transfected cells was plotted (normalized to the vector control).

Kinase assay. In order to examine LIMK activity, Cos-7 cells were transfected with Flag LIMK1 and several Myc-nischarin constructs, or with Myc–β-galactosidase. After 24 h of transfection in DMEM containing 10% FBS, the medium was replaced with serum-free DMEM for another 24 h. Cells were stimulated with 10% FBS for 20 min and lysed in modified RIPA buffer with protease inhibitor cocktail from Roche. The cell lysates were immunoprecipitated with anti-Flag antibody and incubated at 30°C for 1 hour in 50 μl of kinase buffer containing 2 μg of purified cofilin (Cytoskeleton), 25 μM ATP, and 5 μCi of ^{32}P. The reactions were terminated by addition of SDS sample buffer. The samples were electrophoresed on 15% SDS-PAGE gels and stained with Coomassie blue, dried, and exposed to X-ray films.

Inhibition of endogenous nischarin by siRNA. A 21-bp siRNA for human nischarin (shown below) was synthesized by Ambion Inc. (Foster City, CA). A nonspecific control duplex siRNA was synthesized by Dharmacon (Lafayette, CO). To determine the effectiveness of the nischarin siRNA, human breast cancer MCF7 cells were transfected using Lipofectamine 2000 with the plasmid pcDNA-CD4 and with 25 nM siRNA. At 48 h after transfection, cells were detached with trypsin-EDTA, and anti-CD4-coated magnetic beads (Dynal, Invitrogen) were used to enrich the transfected cells by magnetic selection. The cells were lysed in RIPA buffer, and the soluble supernatants were used for immunoblotting. Nischarin levels were determined with an antinischarin antibody. Cell invasion experiments were performed after a similar transfection with nischarin siRNA or control siRNAs (see above), with β-Gal as a marker. The following nischarin duplex siRNA was used: 5′-GGAUCUGGAGGUCUACC UCTT-3′.

RESULTS

Nischarin and LIMK interact in vivo. LIMK stimulates cell invasion, and we have shown that nischarin inhibits cell invasion, suggesting that a functional connection may exist between LIMK and nischarin. We first tested whether intracellular complexes were formed between LIMK and nischarin in vivo by performing coimmunoprecipitation experiments. Cos-7 cells were transiently cotransfected with plasmids expressing Myc-tagged nischarin and HA-tagged LIMK. The LIMK pro-

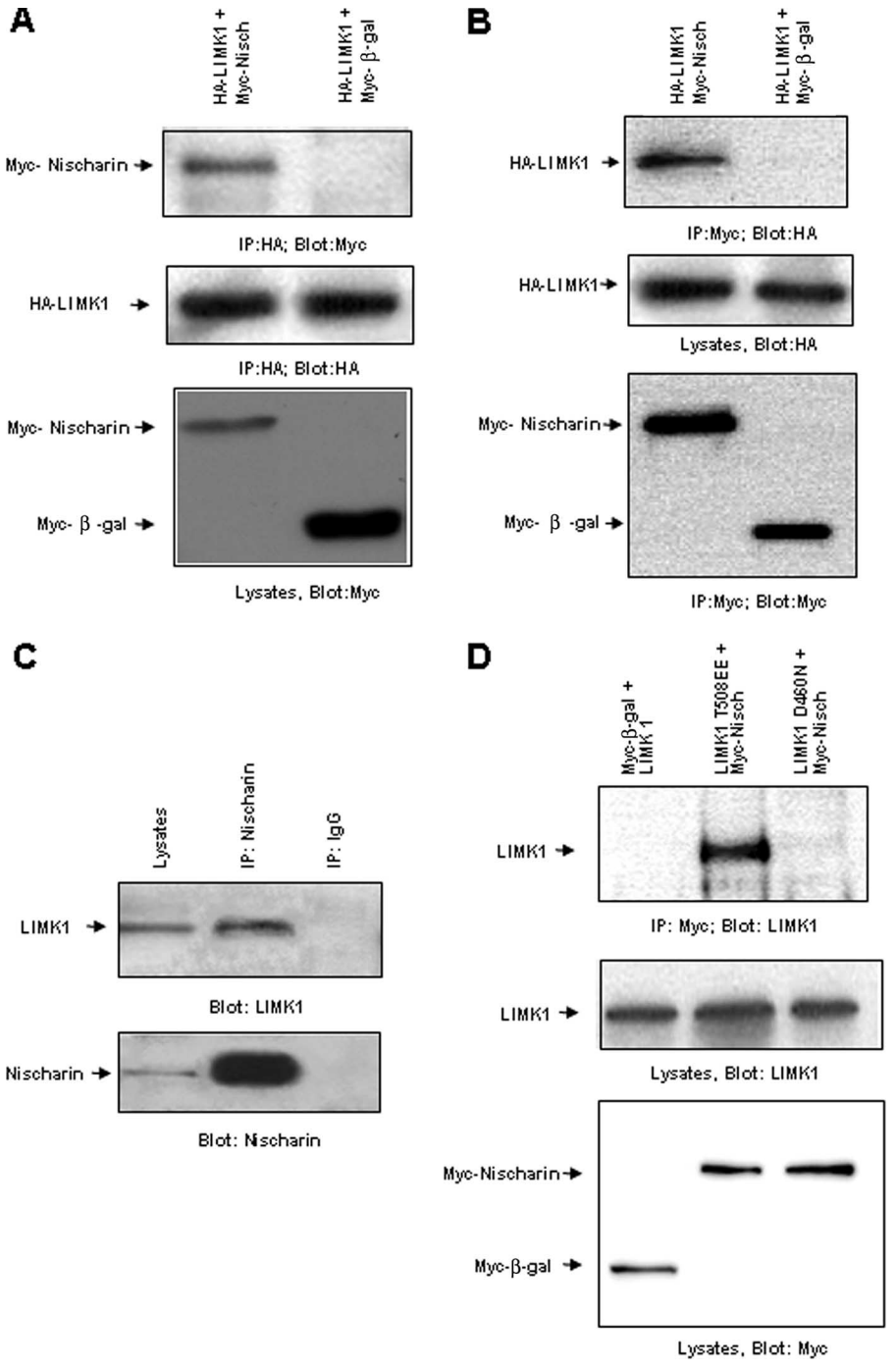

FIG. 1. Nischarin interacts with LIMK in vivo. (A) LIMK immunoprecipitates nischarin. Cos-7 cells were transiently transfected with Myc-nischarin and HA-LIMK1 or Myc-β-galactosidase and HA-LIMK plasmids. The lysates made from these cells were immunoprecipitated (IP) with an anti-HA antibody and immunoblotted with anti-Myc antibody (top panel). HA immunoprecipitates were immunoblotted with anti-HA antibody (middle panel). Input lysates were blotted with an anti-Myc antibody (bottom panel). (B) Nischarin immunoprecipitates LIMK. Cos-7 cells were transfected with HA-LIMK and Myc-nischarin or HA-LIMK and Myc-β-galactosidase plasmids, and the lysates were immunoprecipitated with anti-Myc and blotted with anti-HA antibody (top panel). Myc immunoprecipitates were blotted with anti-Myc (bottom panel). The input lysates were blotted with anti-HA antibody (middle panel). (C) Interactions between endogenous proteins. Lysates of PC12 cells were incubated with either an anti-mouse nischarin monoclonal antibody or mouse immunoglobulin G and precipitated with protein G-Sepharose beads. The nischarin immunoprecipitates were blotted with anti-LIMK1 antibody (top panel) or with antinischarin antibody (bottom panel). The lysates made from PC12 cells are shown in lane 1. (D) Constitutively active LIMK interacts with Myc-nischarin. Myc-nischarin and active LIMK (LIMK 408EE), Myc-nischarin and dominant-negative LIMK (D460N), or LIMK1 and β-Gal were overexpressed in Cos-7 cells. Cell lysates were immunoprecipitated with anti-Myc and immunoblotted with anti-LIMK (top panel). The same lysates (input) were blotted with anti-LIMK (middle panel) or blotted with anti-Myc (bottom panel).

tein was immunoprecipitated using an anti-HA antibody, and associated proteins were detected by Western blot analysis using an anti-Myc antibody. LIMK1 interacted with nischarin but not with the control protein, β-Gal (Fig. 1A). Reciprocal immunoprecipitation of Myc-nischarin with the anti-Myc antibody also coimmunoprecipitated HA-LIMK, while unrelated β-Gal did not bind to HA-LIMK1 (Fig. 1B). These data clearly indicate that LIMK and nischarin interact in vivo.

Endogenous nischarin interacts with endogenous LIMK1. The interactions between overexpressed nischarin and LIMK could result from artifacts of the overexpression of these proteins. We therefore used rat pheochromocytoma cells (PC12) to explore the interaction of endogenous nischarin and LIMK, since both are present in these cells. Nischarin was immunoprecipitated using an antinischarin monoclonal antibody, and a Western blot analysis was performed to detect associated proteins. Nischarin immunoprecipitated LIMK but not immunoglobulin G, indicating that LIMK1 interacts with nischarin in PC12 cells (Fig. 1C). These data suggest that the interaction occurs physiologically.

Nischarin binds to constitutively active LIMK, but not to dominant-negative LIMK. LIMK has a highly basic 11-amino-acid (aa) region (Arg 495 to Arg 506) in its activation loop (20), followed by a threonine (T508), which is similar to a regulatory phosphorylation site in several protein kinases. Replacement of T508 with two glutamic acid residues (T508EE) yields a constitutively active LIMK, while the D460N mutation results in catalytically inactive LIMK (20). We used the constitutively active LIMK and the catalytically inactive (dominant-negative) LIMK in coimmunoprecipitation assays to determine which form of LIMK interacts with nischarin. Cos-7 cells were transiently transfected with active LIMK (T508EE) plus Myc-nischarin or dominant-negative LIMK (D460N) plus nischarin, and lysates were immunoprecipitated with anti-Myc and blotted with LIMK. Active LIMK strongly interacted with Myc-nischarin, while dominant-negative LIMK failed to bind to Myc-nischarin (Fig. 1D), suggesting that LIMK activity is important for the interaction.

Nischarin interacts with LIMK1 via its N-terminal domain. We performed interaction assays with several nischarin truncation mutants to determine which region interacts with LIMK (Fig. 2A). We could coimmunoprecipitate HA-tagged LIMK with full-length Myc-tagged nischarin or its N terminus (aa 1 to 802), but not the C terminus (aa 970 to 1354) in Cos-7 cells (Fig. 2B). We then performed coimmunoprecipitation experiments with four different deletion mutants of nischarin (aa 1 to 217, 218 to 415, 416 to 624, and 625 to 802) and found that only the fragment with aa 416 to 624 (Fig. 2C and D) could interact with LIMK, indicating that other regions of nischarin may not play a role in binding to LIMK.

Nischarin interacts with other LIMK family members. The LIMK family consists of LIMK1 and LIMK2, both of which are serine/threonine kinases that regulate actin dynamics by phosphorylating cofilin. LIMK1 and LIMK2 are predominantly cytoplasmic (24). The major difference between LIMK1 and LIMK2 is that LIMK2 contains a second basic amino acid-rich motif between the PDZ and kinase domains, in addition to the basic amino acid-rich region in the kinase domain (24). Thus, we examined whether nischarin interacted with the other known LIMK family member, LIMK2. Nischarin was coexpressed with HA-LIMK2 in Cos-7 cells. Immunoprecipitation of LIMK2 by its HA tag coprecipitated Myc-nischarin, but not the irrelevant protein Myc-β-galactosidase (Fig. 2E), indicated that the differences in LIMK1 and LIMK2 do not affect their interaction with nischarin.

LIMK1 interacts with nischarin via the PDZ and kinase domains. We next mapped the nischarin binding site in LIMK1 using Flag fusion domains of LIMK1 (Fig. 3A). Cos-7 cells were transiently transfected with Myc-nischarin and several Flag-tagged, truncated LIMK constructs and immunoprecipitated with an anti-Myc antibody to detect associated Flag-tagged proteins. The LIMK1 kinase domain (aa 310 to 633) and the PDZ domain (aa 131 to 290), but not LIM domains 1 and 2 (aa 1 to 130), efficiently interacted with nischarin (Fig. 3B).

Phosphorylation of T508 in LIMK is important for the interaction with nischarin. LIMK activation requires phosphorylation of T508 and multiple subsequent autophosphorylation events (20). Therefore, we examined whether the interaction of nischarin and LIMK required T508 phosphorylation or these subsequent autophosphorylation events. To examine this, we used LIMK1 short (LIMK1s), a splice variant of LIMK1 that lacks autophosphorylation activity on serine residues but can be phosphorylated at T508 (7). Flag-tagged LIMKs was coexpressed with Myc-nischarin in Cos-7 cells (Fig. 3C), and lysates were immunoprecipitated with anti-Myc and Western blotted with an anti-Flag antibody. nischarin was able to interact with LIMKs (Fig. 3B, lane 5), suggesting that the interaction involves phosphorylation on T508 but not serine autophosphorylation.

To further confirm whether T508 phosphorylation is important for nischarin interaction, we coexpressed Flag-LIMK and Myc-nischarin in Cos-7 cells and the lysates were immunoprecipitated with anti-Myc followed by immunoblotting with phospho-LIMK antibody. As shown in Fig. 4A, LIMK associated with nischarin is indeed phosphorylated on threonine 508. It is important to note that lower amounts of nischarin needed to be used here in order to find an association of phosphorylated LIMK with nischarin (as noted below, nischarin blocks LIMK phosphorylation). The lysates made from these cells were also immunoblotted with phospho-LIMK1 antibody, and the results revealed that nischarin reduced LIMK phosphorylation (Fig. 4A). These data clearly indicate that nischarin has stronger affinity toward phosphorylated form of LIMK.

Our earlier study showed that nischarin inhibits PAK activation (6), and PAK is known to phosphorylate LIMK (20). To determine whether the association of nischarin with LIMK depends on PAK activation, we suppressed PAK activation with a plasmid that expresses the autoinhibitory domain of PAK (known to affect all group1 PAK family members [63]) and with a plasmid that expresses kinase dead (K299R) PAK (known to act as a dominant negative of PAK [20]) and examined the association between LIMK and nischarin. Interestingly, nischarin binds to LIMK regardless of PAK activation (Fig. 4B, lanes 3 and 4; PAK was inhibited using K299R and PAK AID plasmids), suggesting that the interaction of nischarin with LIMK is likely to be independent of PAK's association with nischarin.

Further, we examined whether nischarin, LIMK, and PAK exist in a complex. Flag LIMK, V5-PAK, and Myc-nischarin

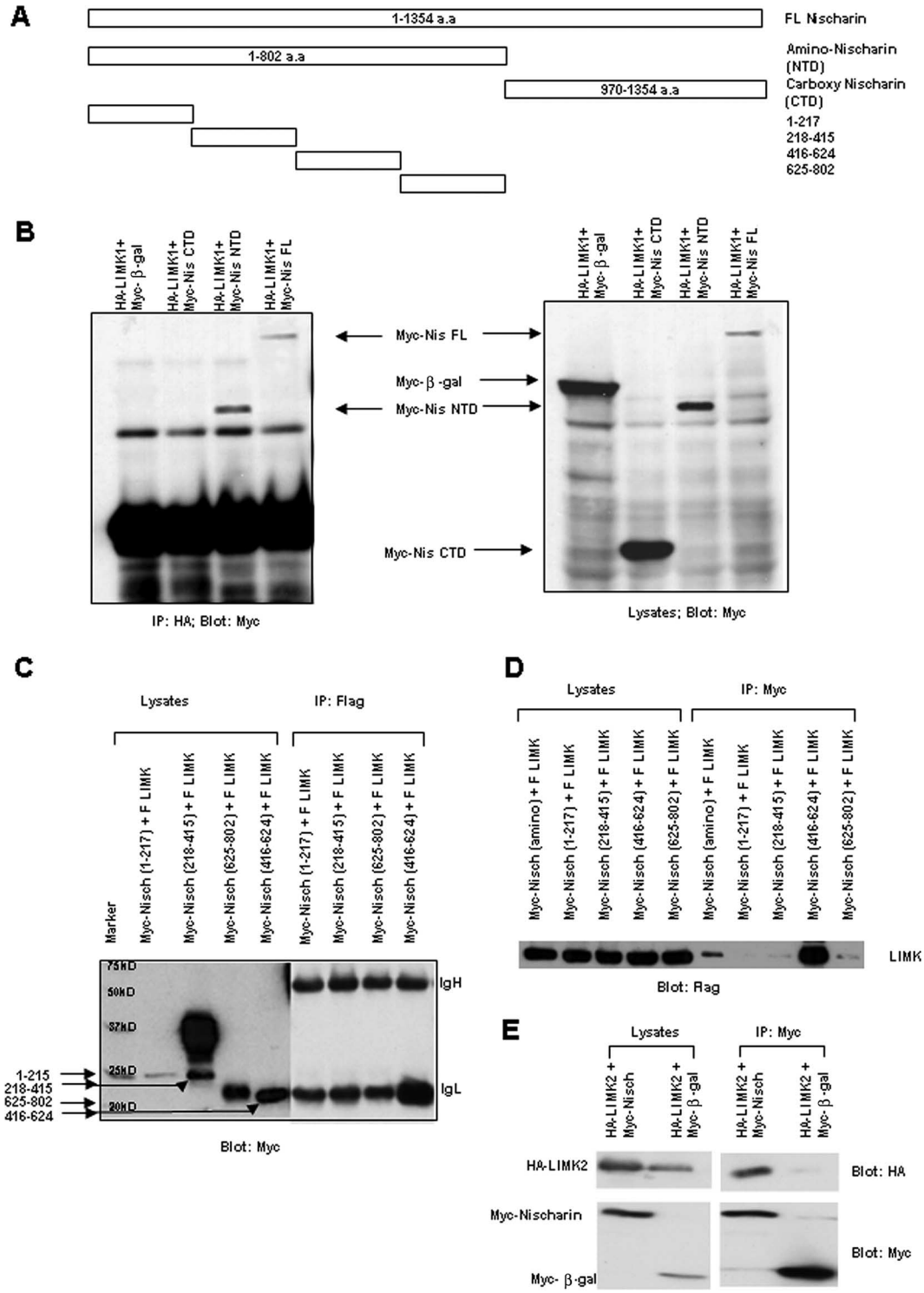

FIG. 2. Identification of the minimal region of nischarin that retains LIMK binding. (A) Schematic of full-length nischarin and truncations used in the study. (B) An N-terminal fragment of nischarin interacts with LIMK. Cos-7 cells were transiently transfected with full-length Myc-nischarin plus HA-LIMK, the N-terminal region of Myc-nischarin and HA-LIMK, the C-terminal region of Myc-nischarin and HA-LIMK, or Myc–β-Gal and HA-LIMK. Cell lysates were immunoprecipitated (IP) with an anti-HA antibody and immunoblotted with an anti-Myc antibody (left panel). The lysates were immunoblotted with an anti-Myc antibody (right panel). (C) A nischarin fragment of aa 416 to 624 is sufficient for interaction with LIMK. Myc-nischarin (amino) plus Flag-LIMK, Myc-nischarin (aa 1 to 217) plus Flag-LIMK, Myc-nischarin (aa 218 to 415) plus Flag-LIMK, Myc-nischarin (aa 416 to 624) plus Flag-LIMK, or Myc-nischarin (aa 625 to 802) plus Flag-LIMK were expressed in Cos-7 cells, immunoprecipitated with an anti-Flag antibody, and blotted with an anti-Myc antibody. Lysates also were immunoblotted with an anti-Myc antibody. (D) LIMK interacts with a Myc-nischarin (aa 416 to 624) fragment. The same lysates described for panel C were immunoprecipitated with anti-Myc and blotted with an anti-Flag antibody. (E) Nischarin interacts with LIMK2. HA-LIMK2 plus Myc-nischarin or HA-LIMK2 plus Myc–β-Gal were transfected into Cos-7 cells, and the lysates made from these cells were immunoprecipitated with anti-Myc and blotted with anti-HA (right top panel). The same blot was stripped and blotted with anti-Myc (right bottom panel). The left panel shows the Western blot of lysates from the same cells blotted with anti-HA (top panel) and blotted with anti-Myc (bottom panel) (lanes 1 and 2).

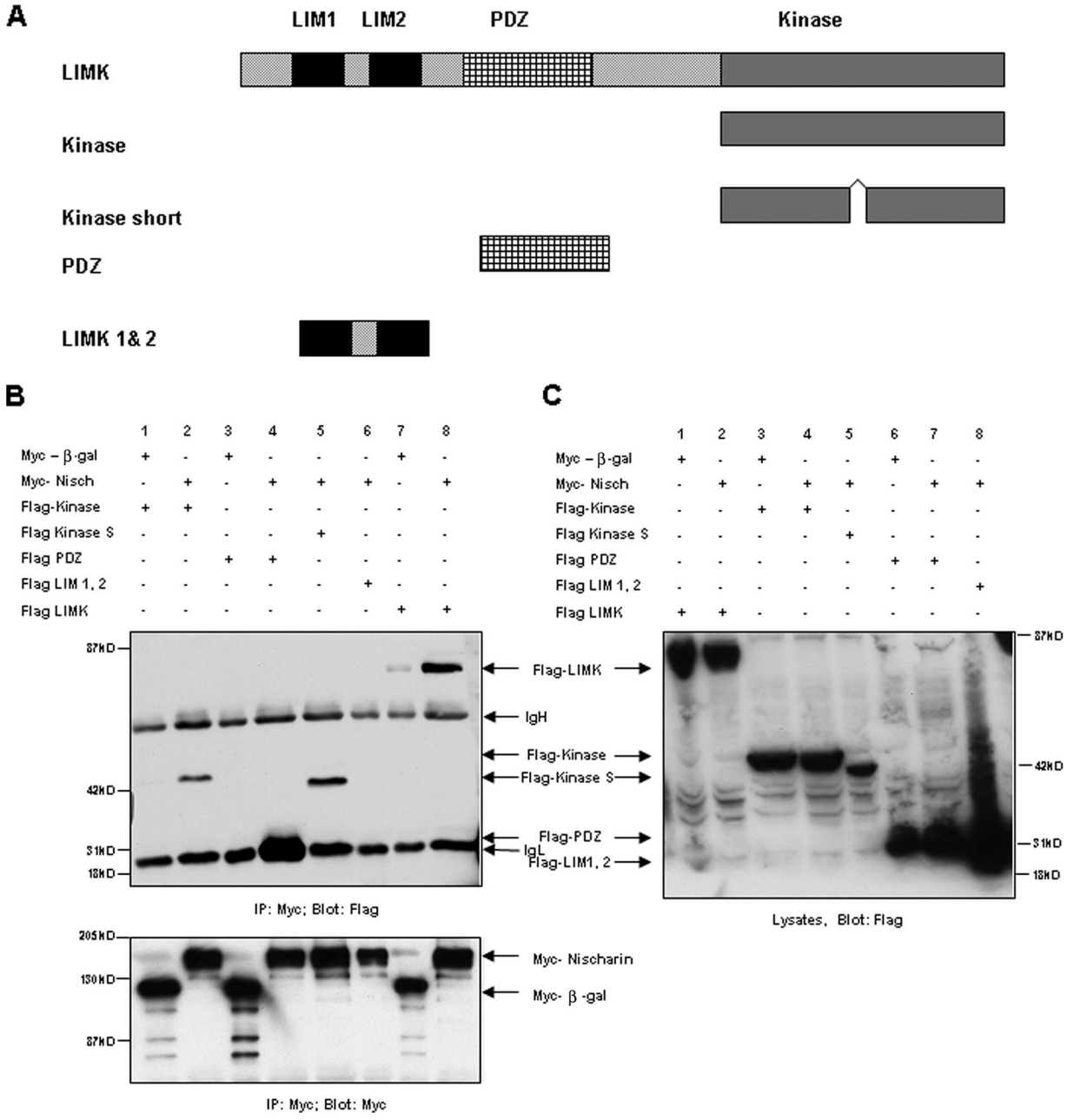

FIG. 3. Identification of domains of LIMK that interact with nischarin. (A) Schematic of LIMK truncations. (B) Nischarin interacts with the PDZ and kinase domains of LIMK. Cos-7 cells were transiently transfected with Flag-LIMK and Myc-nischarin, Flag-LIM domains 1 and 2 plus Myc-nischarin, Flag-PDZ plus Myc-nischarin, Flag kinase domain plus Myc-nischarin, Flag kinase domain short plus Myc nischarin, or Flag-LIMK plus Myc–β-Gal, Flag-PDZ plus Myc–β-Gal, or Flag kinase domain plus Myc–β-galactosidase. The lysates were immunoprecipitated with an anti-Myc antibody and blotted with an anti-Flag antibody (top panel). The bottom panel shows the expression levels of nischarin and β-Gal. (C) Expression levels of Flag-LIMK proteins. The same lysates described for panel B were immunoblotted with anti-Flag antibody.

expressed in Cos-7 cells was immunoprecipitated with anti-Myc and blotted with V5 or Flag antibodies. As depicted in Fig. 4C, nischarin immunoprecipitates contained both PAK and LIMK. Although LIMK interaction with nischarin is independent of PAK's association, complexes containing all three proteins exist, suggesting a likely possibility of simultaneous association between nischarin, PAK, and LIMK.

The data from the Fig. 3 indicate that LIMK T508 phosphorylation is likely to be important in the interaction with nischarin. To further confirm the importance of threonine 508 phosphorylation, we expressed a Flag-tagged LIMK mutant of threonine 508 to valine (T508V), which reduced LIMK activity (20), along with Myc-tagged nischarin and performed coimmunoprecipitation assays. As shown in Fig. 4D, nischarin is unable to bind LIMK T508V while it is associated with wild-type (WT) LIMK, confirming the interaction depends on T508 phosphorylation. These data suggest LIMK kinase activity per se is not required, but the initial threonine phosphorylation may be crucial for the interaction.

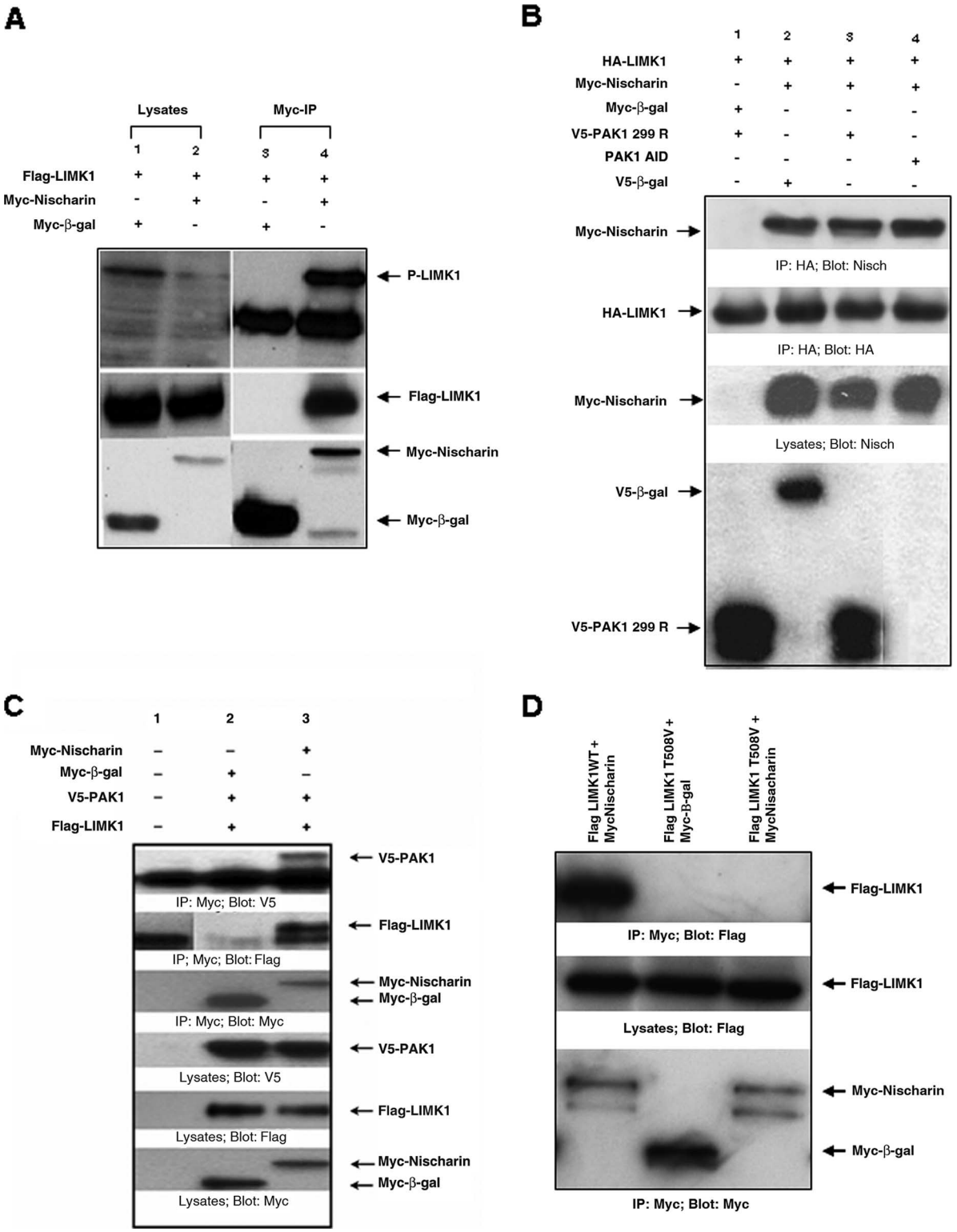

FIG. 4. Nischarin binds to threonine 508-phosphorylated LIMK. (A) Nischarin binds to T508-phosphorylated LIMK. Cos-7 cells were transiently transfected with 2 μg of Flag-LIMK and 1 μg of full-length Myc-nischarin or Myc–β-galactosidase. After 48 h of transfection, cell lysates were prepared and immunoprecipitated with anti-Myc antibody. The immunoprecipitates (IP) and the lysates were run on a SDS-PAGE gel and immunoblotted (IB) with anti-phospho-LIMK antibody (top panel). Left panel (lanes 1 and 2), lysates; right panel (lanes 3 and 4), Myc immunoprecipitates. The middle panel shows the expression of Flag-LIMK, and the bottom panel shows the expression of Myc-tagged proteins. The quantitative data from three different experiments indicated that the inhibition of P-LIMK by nischarin was 56% in the lysates. (B) Inhibition of PAK does not have any effect on LIMK binding with nischarin. The plasmids expressing HA-LIMK1, Myc-nischarin with V5–β-Gal, V5-PAK 299R, or PAK AID were transfected into Cos-7 cells, and the lysates made from these cells were immunoprecipitated with anti-HA antibody and

To confirm whether nischarin and LIMK interact directly in vitro, we expressed nischarin as a GST-fusion protein in a pGEX 4T2 vector. GST-nischarin (aa 33 to 588) was immobilized onto a glutathione-agarose matrix and incubated with purified His-tagged LIMK1 protein (purified from insect cells [Invitrogen]). The proteins bound to the glutathione matrix were separated by SDS-PAGE and blotted with an anti-His antibody. Consistent with our in vivo data, the GST-nischarin protein, but not GST alone, was able to interact with purified LIMK1 protein (data not shown) in vitro. Protein production outside mammalian cells reduces the chances that other binding proteins mediate this interaction, suggesting that the interaction is direct.

Nischarin negatively regulates LIMK activity. We next examined the effect of nischarin on the ability of LIMK1 to phosphorylate its downstream effector, cofilin, using an in vitro kinase assay. Flag-tagged LIMK1 and Myc-tagged nischarin were expressed in Cos-7 cells, immunoprecipitated with an anti-Flag antibody, and then used in an in vitro kinase assay, with cofilin as the substrate. As a control, β-Gal was expressed along with Flag-LIMK. LIMK was able to phosphorylate cofilin in the presence of serum. Full-length, but not the N or C terminus of nischarin (Fig. 5A and data not shown), inhibited LIMK-mediated phosphorylation of cofilin. Furthermore, we examined if nischarin affected LIMK in exponentially growing cells. After transfection with the LIMK and nischarin, cells were left in serum medium for 24 hours, and in vitro kinase assays were performed. As illustrated in Fig. 5B, nischarin has a modest inhibitory effect on cofilin phosphorylation, suggesting that nischarin effect lasts only for a short period of time after serum stimulation. Consistent with this, nischarin had a stronger inhibitory effect (Fig. 5A) when cells were stimulated with serum for 30 minutes. Since it is known that ROCK activates LIMK, we examined whether nischarin affects ROCK-mediated LIMK activation. Overexpression of constitutively active ROCK-stimulated LIMK and nischarin was able to inhibit the LIMK activation (data not shown), indicating that nischarin blocks ROCK-mediated LIMK activation as well. It is not surprising to see an effect on ROCK signaling as well, because both PAK and ROCK phosphorylate LIMK on threonine 508, and as described below nischarin affects T508 phosphorylation. In summary, these experiments revealed that nischarin inhibits activation of LIMK.

The above data in Fig. 4 indicate that LIMK T508 is crucial for interaction with nischarin. To further confirm that phosphorylation of LIMK is indeed important for its binding to nischarin, the lysates made from cells expressing Flag-LIMK and Myc-nischarin were treated with γ-phosphatase, immunoprecipitated with anti-Myc, and immunoblotted with phospho-LIMK antibody (Fig. 5C). Consistent with the data in Fig. 4, nischarin was only able to bind to phospho-LIMK (in phosphatase-untreated cells). As expected no phospho-LIMK was present in phosphatase-treated cells, and thus nischarin did not immunoprecipitate any phospho-LIMK (Fig. 5C). Overall, these data clearly indicate that phosphorylation of LIMK is important for the interaction between nischarin and LIMK.

Nischarin inhibits threonine 508 phosphorylation of LIMK. PAK1 phosphorylates LIMK1, and nischarin inhibits PAK kinase activity. Since PAK phosphorylates LIMK at T508, which is required for interaction with nischarin, we investigated whether nischarin specifically blocked T508 phosphorylation. We transiently coexpressed V5-PAK1 (T423E) and HA-LIMK1, as well as either Myc-nischarin or Myc vector control, into Cos-7 cells. Immunoprecipitated LIMK1 proteins were blotted with an antibody that specifically detects T508 phosphorylation (p-LIMK). Overexpression of PAK1 increased phosphorylation of LIMK1, while coexpression of full-length nischarin blocked this increase in phosphorylation (Fig. 6A, lanes 2 and 3). Coexpression of vector alone had no effect, and LIMK phosphorylation was not detected in the absence of active PAK (Fig. 6A, lane 1). As described below, nischarin could bind to active PAK and might not allow PAK to phosphorylate LIMK, and this could cause reduction of LIMK phosphorylation here. To rule out this possibility, we examined the effect of nischarin on serum-stimulated LIMK phosphorylation (LIMK can be activated by non-PAK-mediated signaling events). Serum stimulation increased phosphorylation of LIMK1, while coexpression of full-length nischarin blocked this increase in phosphorylation (Fig. 6A, lanes 4 and 5). These data clearly indicate that nischarin downregulates T508 phosphorylation of LIMK.

Nischarin inhibits endogenous cofilin phosphorylation. LIMK phosphorylates cofilin, leading to inactivation. Since cofilin is a downstream effector of LIMK, we examined whether nischarin-mediated inhibition of LIMK affected phosphorylation of endogenous cofilin. Although we showed this regulation in the kinase assay (Fig. 5A), we wished to see the effect in vivo. We expressed HA-LIMK1 plus Myc-β-galactosidase or HA-LIMK1 plus Myc-nischarin separately in MCF7 cells, and the lysates were immunoblotted with a phospho-cofilin antibody. Serum starvation did not stimulate cofilin phosphorylation (Fig. 6B, lane 1). Interestingly, nischarin was able to reduce cofilin phosphorylation in serum-stimulated cells, while β-Gal had no effect (Fig. 6B, lanes 2 and 3).

These experiments demonstrated that nischarin blocks serum-mediated activation of LIMK1. However, they did not distinguish between direct effects on LIMK1 itself and blockade of the signaling pathway leading to LIMK activation. To

immunoblotted with antinischarin antibody (top panel). The immunoprecipitates were also immunoblotted with anti-HA antibody (second panel from the top). To confirm the expression of nischarin- and V5-tagged proteins, the lysates were blotted with antinischarin antibody (third panel from the top) and blotted with anti-V5 (bottom panel). (C) Nischarin, PAK, and LIMK exist in a complex together. V5-PAK and Flag-LIMK were expressed in Cos-7 cells with Myc-nischarin or its control, Myc–β-Gal. The Myc immunoprecipitates were immunoblotted (IB) with anti-V5 (top panel), anti-Flag (second from the top panel), and anti-Myc (third from the top) antibody. The bottom three panels show the expression of V5 PAK1, Flag-LIMK1, and Myc-tagged proteins in the lysates. Lane 1, untransfected Cos-7 cells. (D) Nischarin does not bind to LIMK T508V. Flag-LIMK plus Myc nischarin, Flag-LIMK T508V plus Myc–β-Gal, or Flag-LIMK T508V plus Myc-nischarin were transfected into Cos-7 cells, and the lysates made from these cells were immunoprecipitated with anti-Myc and immunoblotted with anti-Flag (top panel) and anti-Myc (bottom panel). Also, the lysates were blotted with anti-Flag antibody (middle panel).

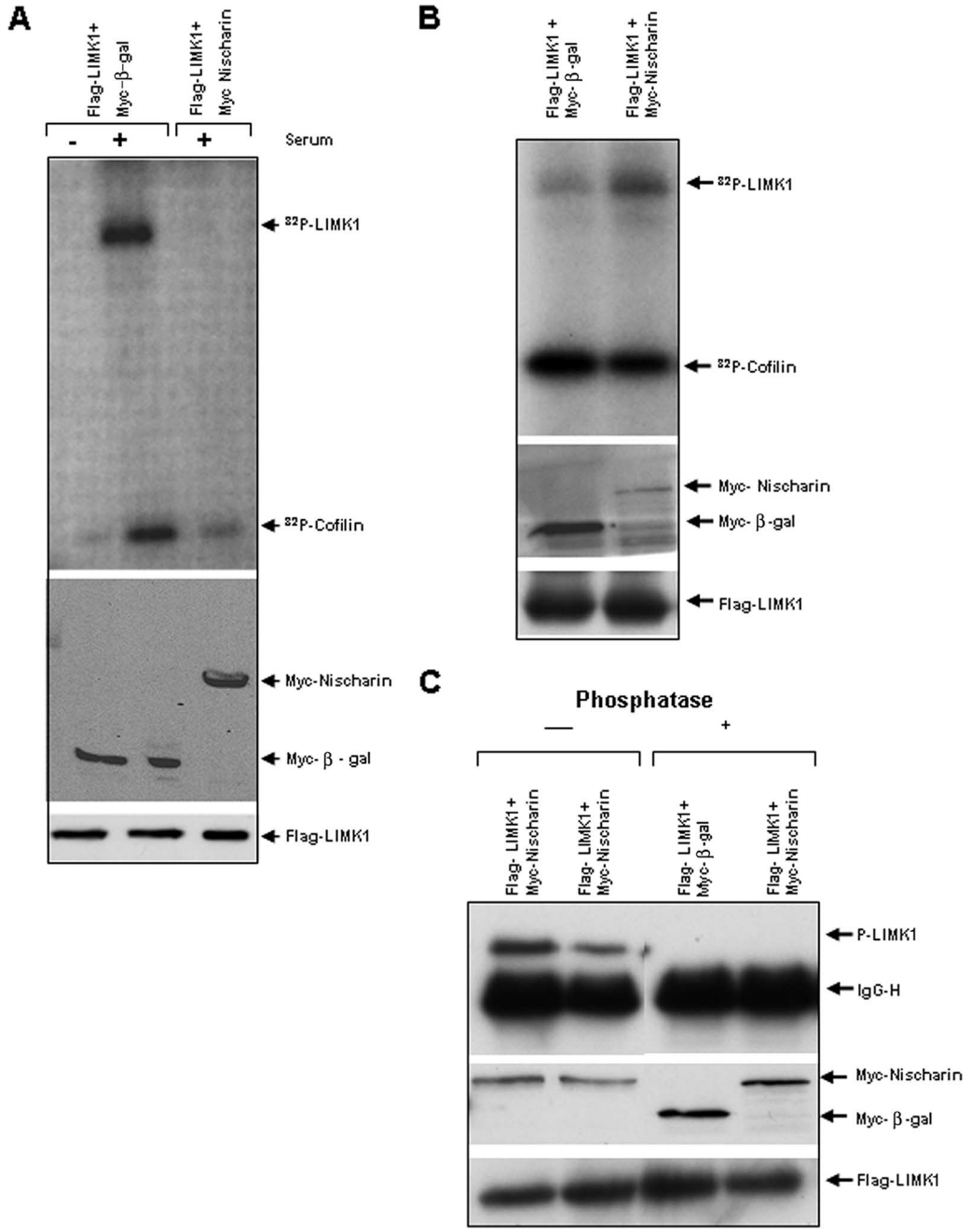

FIG. 5. Nischarin inhibits LIMK activation. (A) Kinase activity in serum-starved and serum-stimulated cells. Cos-7 cells were transfected with Flag-LIMK plus Myc-nischarin or Flag-LIMK plus Myc–β-Gal. After 24 h of transfection, the cells were serum starved for 24 h and stimulated with serum for 30 min. Cell lysates were immunoprecipitated with an anti-Flag antibody. The Flag immunoprecipitates were used for in vitro LIMK assays using cofilin as a substrate. The same lysates were blotted with an anti-Myc antibody (middle panel) or anti-Flag antibody (bottom panel). (B) Kinase activity in exponentially growing cells. Cos-7 cells were transfected with Flag-LIMK plus Myc–β-Gal or Flag-LIMK plus Myc-nischarin. After 24 h of transfection, the cells were lysed and immunoprecipitated with an anti-Flag antibody, and in vitro kinase assays were performed using cofilin as a substrate. The same lysates were blotted with an anti-Myc antibody (middle panel) or anti-Flag antibody (bottom panel). (C) Phosphatase treatment blocks the interaction between nischarin and LIMK. The lysates made from Cos-7 cells expressing Flag-LIMK plus Myc-nischarin and Flag-LIMK plus Myc–β-Gal were treated with γ-phosphatase, immunoprecipitated with anti-Myc antibody, immunoblotted with phospho-specific anti-LIMK antibody (top panel), and the lysates were immunoblotted with anti-Flag (bottom) or anti-Myc antibody (middle). Two different Myc-nischarin plus Flag-LIMK-expressing cell lysates were not treated with phosphatase (left panel).

investigate whether nischarin acts directly on LIMK, a constitutive and partially activated form of LIMK (LIMK-T508EE) was used in cofilin phosphorylation experiments. Thus, we expressed LIMK T508EE plus Myc-β-Gal or LIMK T508EE plus Myc-nischarin in MCF7 cells, and the lysates were probed with phospho-cofilin. Consistent with the serum stimulation data, nischarin reduced the cofilin phosphorylation stimulated by active LIMK (Fig. 6B, lanes 4 and 5). Since nischarin affects

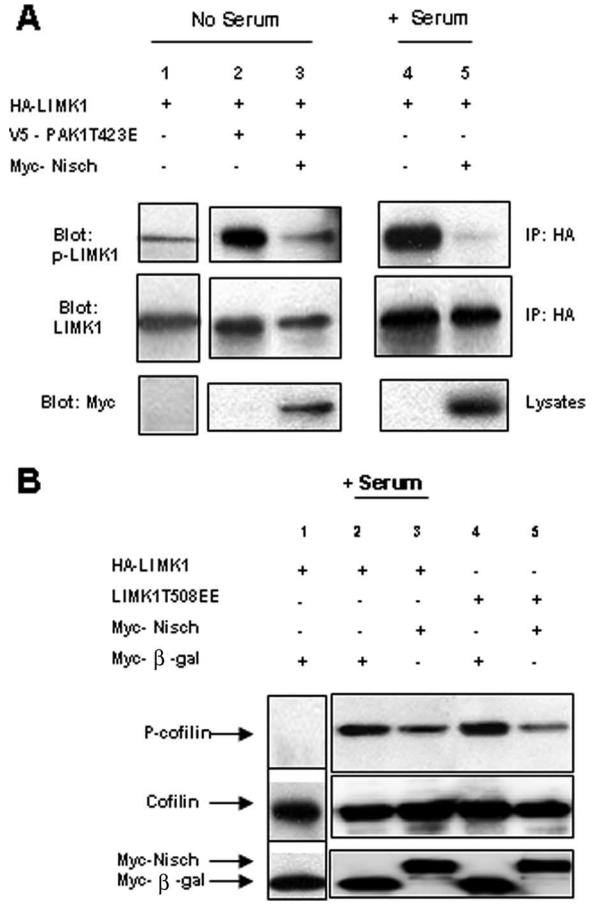

FIG. 6. LIM kinase and cofilin phosphorylation. (A) LIMK phosphorylation. Cos-7 cells were transfected with HA-LIMK1 plus Myc vector control, V5-PAK T423E plus HA LIMK plus Myc vector control, or V5-PAKT423E plus HA-LIMK plus Myc-nischarin and serum starved for 24 h. The lysates made from these cells were immunoprecipitated (IP) with anti-HA antibody and blotted with a phospho-specific anti-LIMK antibody (top panel, lanes 1 to 3). The same blot was stripped and reblotted with anti-LIMK antibody (middle panel). The bottom panel shows the expression of Myc-nischarin. Plasmids expressing HA LIMK V5-PAK plus Myc vector or HA LIMK plus V5 PAK plus Myc-nischarin (or vector control) were transfected into Cos-7 cells and stimulated with serum for 30 min. Cell lysates were immunoprecipitated with an anti-HA antibody and blotted with a phospho-specific anti-LIMK antibody (top panel, lanes 4 and 5). The same blot was stripped and blotted with anti-HA antibody (middle panel, lanes 3 and 4). The bottom panel shows the expression of Myc-nischarin. (B) Nischarin inhibits cofilin phosphorylation. MCF7 cells were transfected with HA-LIMK plus β-Gal, HA-LIMK plus Myc nischarin, LIMK T508EE plus Myc–β-Gal, or LIMK T508EE plus Myc-nischarin for 48 h. The samples in lanes 1, 4, and 5 were serum starved, and the samples in lanes 2 and 3 were stimulated with serum. The lysates made from these cells were run on a 15% gel and blotted with anti-phospho-cofilin or total cofilin antibody.

constitutively active LIMK, the effect of nischarin on LIMK is likely to be direct. It is known that PAK does not have any effect on active LIMK (20), and thus nischarin seems to inhibit directly the ability of LIMK to phosphorylate cofilin, suggesting that inhibition of LIMK1 kinase activity might be independent of nischarin's effect on PAK. It is interesting that nischarin has a stronger effect on LIMK phosphorylation than cofilin phosphorylation, and the reasons for this are unclear. However, similar situations have been reported (41, 61). Overall, these data indicate nischarin decreases LIMK activation and blocks LIMK activity, which in turn regulates cofilin phosphorylation.

Suppression of LIMK1-induced cell invasion by nischarin. LIMK1 regulates cell invasion, so we hypothesized that nischarin-mediated inhibition of LIMK1 could inhibit cell invasion. We therefore examined the effects of nischarin on invasion stimulated by an active PAK (PAK1-T423E). MCF7 cells were transfected with PAK1-T423E and wild-type LIMK1 alone or were also cotransfected with nischarin. Subsequent to transfection, cell invasion assays were performed as described elsewhere (3). PAK1-T423E stimulated invasion in the MCF7 cells, and this was strongly inhibited by overexpression of nischarin (Fig. 7A), and the difference was statistically significant. Since nischarin also inhibits LIMK1 activity, it was important to determine whether nischarin suppresses cell invasion by inhibiting LIMK1 directly. To address this question, cells were transfected with an active form of LIMK1 (T508EE), with or without nischarin. LIMK1-T508EE dramatically stimulated cell invasion, and nischarin strongly inhibited this invasion (Fig. 7A); the inhibition was statistically significant. These results are consistent with the findings that nischarin binds to and inhibits both PAK1 (6) and LIMK1.

To better understand the role of endogenous nischarin, we examined the effect of knocking down nischarin expression on cell invasion. MCF7 breast carcinoma cells were transfected with a nischarin siRNA or control siRNA. The nischarin siRNA substantially reduced the expression of nischarin, whereas the control siRNA had no effect (Fig. 7C). Also, the nischarin siRNA, but not the control siRNA, dramatically stimulated cell invasion, while full-length nischarin inhibited cell invasion (Fig. 7B). These data suggest that inhibition of endogenous nischarin can stimulate cell invasion, possibly by enhanced phosphorylation of LIMK/cofilin.

Suppression of nischarin stimulates cofilin phosphorylation. Since overexpression of nischarin inhibits cofilin phosphorylation, we examined whether suppression of endogenous nischarin would affect cofilin phosphorylation. MCF7 cells were transfected with nischarin siRNA, or with a control siRNA, and phosphorylation of cofilin was examined. The nischarin siRNA, but not the control, stimulated phosphorylation of endogenous cofilin (Fig. 7C), suggesting that reduced levels of endogenous nischarin promote cell invasion by inhibiting the activation of cofilin. These data strongly suggest that nischarin's effects on cell invasion are mediated through LIMK and cofilin.

Nischarin siRNA stimulates actin reorganization. Since nischarin siRNA stimulated cofilin phosphorylation and cell invasion in MCF7 cells, we wished to determine whether this was accompanied by changes in actin and tubulin organization. Nischarin siRNA and its control siRNA were transfected into MCF7 cells. As a marker, GFP plasmid was introduced in every condition. After 24 h, transfected cells were plated on fibronectin substrata and the structures of F-actin and microtubules were visualized by confocal microscopy. Phalloidin staining indicated that many of the transfected MCF7 cells had a very peculiar phenotype, with several branched projections that contained actin (Fig. 8A), rather than as the widely spread single lamellipodium in one direction in adherent cells. Al-

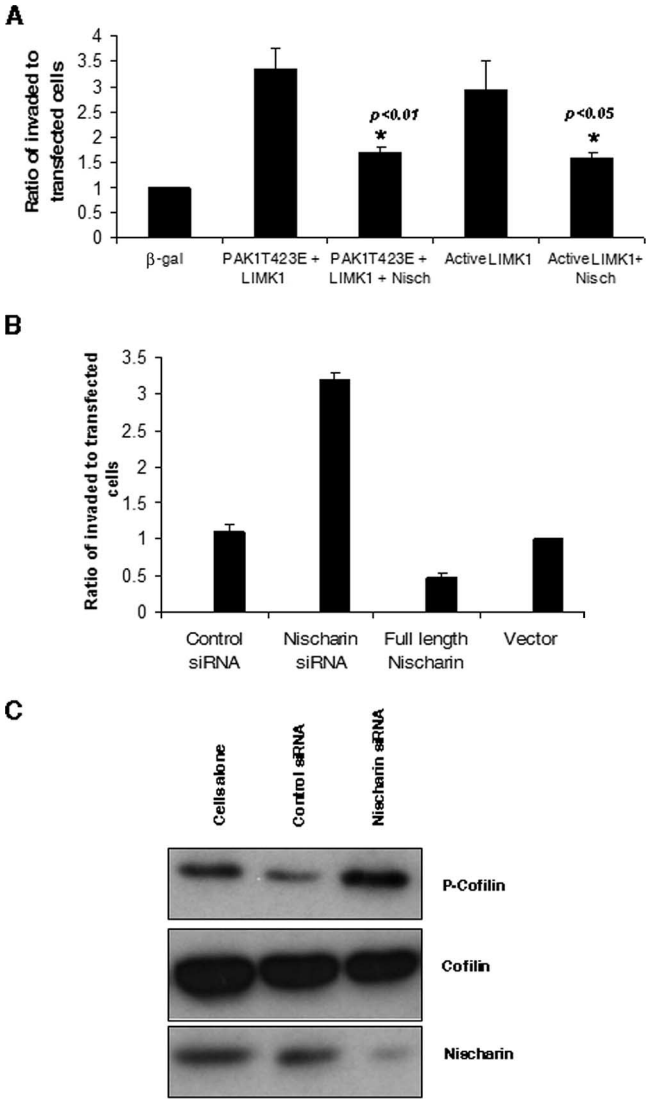

FIG. 7. Nischarin inhibits LIMK-driven cell invasion. (A) Nischarin blocks cell invasion. MCF7 cells were transiently transfected with LIMK (T508EE) plus Myc vector, LIMK (T508EE) plus Myc-nischarin, T423E PAK plus WT LIMK plus vector control, or T423E PAK plus WT LIMK plus Myc-nischarin. After 48 h of transfection, in vitro cell invasion assays were performed as described elsewhere (3). The amount of DNA was normalized. β-Gal was introduced in every transfection to visualize the cells. *, the difference between the PAK plus LIMK and PAK plus LIMK plus nischarin samples was significant at the 0.01 level, and the difference between active LIMK and active LIMK plus nischarin was significant at the 0.05 level. (B) Nischarin siRNA stimulates cell invasion. MCF7 cells were transfected with nischarin siRNA, a control siRNA, full-length nischarin plasmid, or a control plasmid along with a β-Gal plasmid. After 48 h of transfection, cells migrating through matrigel were counted by staining for β-Gal as described previously (3). (C) Nischarin siRNA stimulates endogenous cofilin phosphorylation. MCF7 cells were transfected with 50 nM of nischarin siRNA or control siRNA. After 48 h of transfection, the cells were lysed and immunoblotted with phospho-cofilin antibody (upper panel). The same blot was stripped and blotted with anticofilin (middle panel) and antinischarin (bottom panel) antibodies.

though this is not a universal phenotype, more than 60% of MCF7 cells transfected with nischarin siRNA showed branched projection actin structures (Fig. 8B). In contrast, only 1 to 2% of control siRNA transfected cells had this phenotype. Tubulin staining did not reveal any significant difference between nischarin and its control siRNA (Fig. 8C). It is known that cofilin activity plays an important role in the directionality of migration and random migration is a consequence of formation of multiple lamella and, thus, it is likely nischarin siRNA stimulates random migration and in turn stimulates invasion.

DISCUSSION

Cell invasion is an important cellular event that involves several signal transduction pathways (54). Cancer cells use their migratory potential to invade neighboring tissues and eventually to metastasize to different organs (54). The actin cytoskeleton plays a major role in the regulation of cell migration (55). The signals that regulate the actin cytoskeleton are upregulated in cancer cells to enhance cell migration and, in turn, cell invasion. LIMK and cofilin are important regulators of actin cytoskeleton (55). Despite intensive studies on the proteins that regulate cell invasion, relatively little is known about how they interact with other cellular components to orchestrate these events. Nischarin is a potential inhibitor of LIMK activation and cell invasion, and thus further understanding of this interaction may provide insight into the regulation of cell invasion.

LIMK is a cytoskeletal protein that regulates the actin cytoskeleton through cofilin, an actin-depolymerizing protein, to promote cell invasion (8, 61). LIMK is activated by Rho, Rac, Cdc42, PAK, and ROCK (10) and inhibited by Par3 (for LIMK2) (13), BMPR II (21), and LATS 1 (60), but none of these inhibitors affects cell invasion. Here for the first time we report that nischarin is a negative regulator of LIMK1, and we identify a signaling pathway in breast cancer cells by which this regulation affects cell invasion. LIMK is a serine/threonine kinase that consists of two LIM domains, a PDZ domain, and a kinase domain (10). Deletion of the N-terminal region of LIMK, which contains the LIM and PDZ domains, increases LIMK activity in vitro (7), suggesting that the N terminus acts as a constitutive inhibitor that needs to be inactivated for full LIMK activity. Several proteins associate with LIMK, mostly at the LIM and PDZ domains, including neuregulin (51), protein kinase C (31), BMPRII (21), LATS1 (60), and PAK4 (14), with one protein, SSH 1L, binding to the kinase domain (46). Nischarin is the first protein, to our knowledge, that binds to both the PDZ domain and the kinase domain of LIMK. Thus, nischarin is a novel negative regulator of LIMK1.

Nischarin was first identified as an α5-integrin-interacting protein (4). The amino-terminal domain (aa 1 to 802) of nischarin binds to LIMK, with a region from aa 416 to 624 in the N terminus being sufficient for this interaction. Interestingly, the N terminus also binds to integrin α5 (4), PAK (6), and Rac (43), and it is not clear how so many proteins bind to the same region. Nischarin may bind to several proteins to regulate different biological functions, a situation somewhat analogous to 14-3-3 binding to Raf kinase, IRS, MEK kinases, Cdc25, and others (reviewed in reference 2).

Our data show that nischarin binds tightly to active LIMK but not the dominant negative LIMK, suggesting that nischarin's interaction depends on LIMK activity. LIMK activity depends on phosphorylation of T508 in the kinase domain of

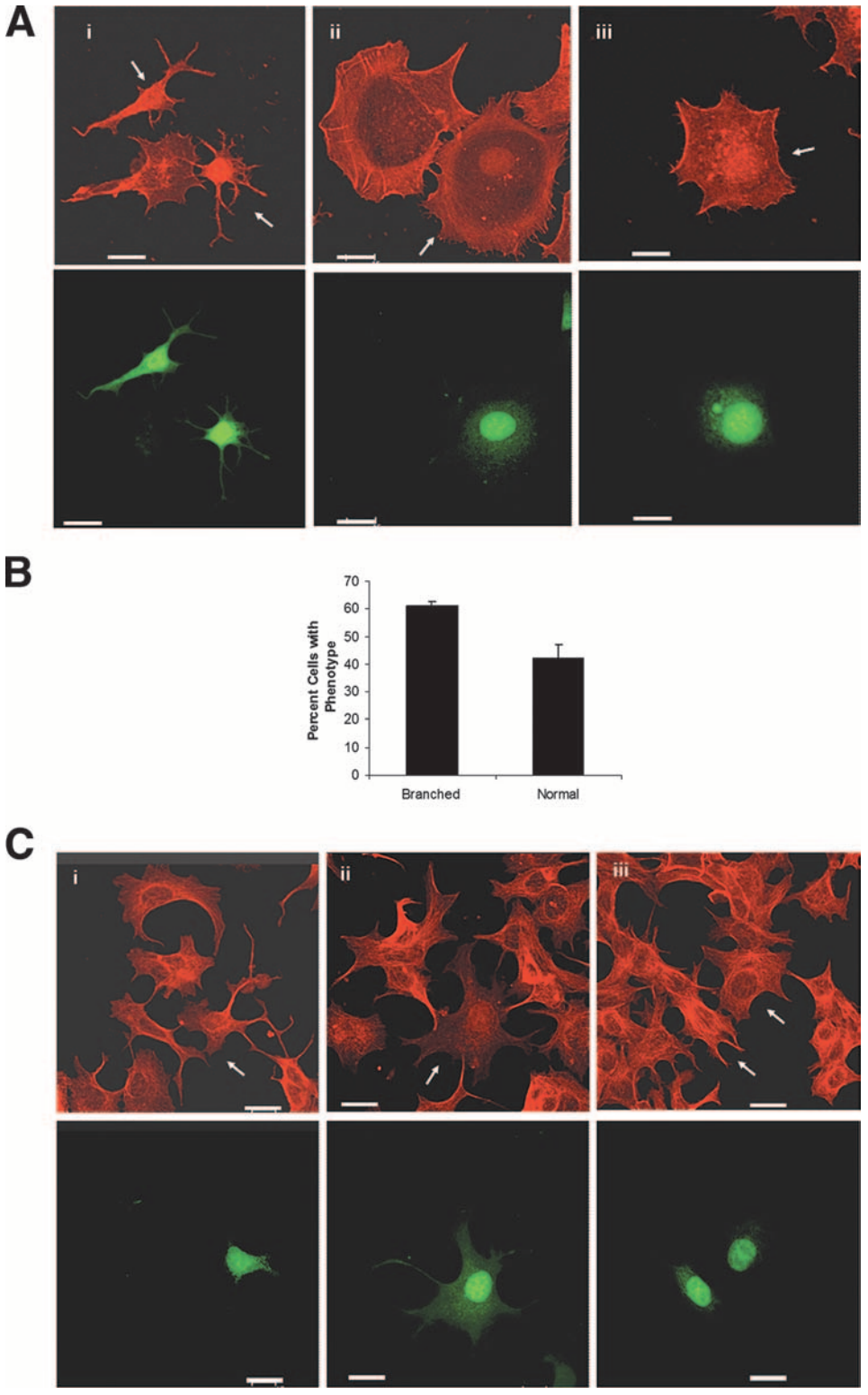

FIG. 8. Effects of nischarin siRNA on F-actin and microtubules. (A) Effect of nischarin siRNA on actin. MCF7 cells were transfected with GFP plus nischarin siRNA or GFP plus control siRNA. The transfected cells were plated on fibronectin-coated coverslips for 3 hours, stained with phallodin, and observed using a Leica confocal scanning microscope. Phalloidin staining is shown in red (top); GFP is shown in green (bottom). (i) Cells transfected with nischarin siRNA plus GFP; (ii) cells transfected with GFP alone; (iii) cells transfected with control siRNA plus GFP. Transfected cells are indicated by the arrows. Bar, 15 μm. (B) The effects of nischarin siRNA on actin are statistically significant. The data (details as described for panel A) from three different experiments were used for this analysis. The cells that have a branched phenotype were compared with the normal phenotype, and the percent control data was plotted. *, the difference between the branched phenotype and normal was significant at the 0.01 level. (C) Effect of nischarin siRNA on tubulin. MCF7 cells were transfected with GFP plus nischarin siRNA or GFP plus control siRNA. The transfected cells were plated on fibronectin-coated coverslips for 3 hours, stained with tubulin, and observed using a Leica confocal scanning microscope. Tubulin staining is shown in red (top); GFP is shown in green (bottom). (i) Cells transfected with nischarin siRNA plus GFP; (ii) cells transfected with GFP alone; (iii) cells transfected with control siRNA plus GFP. Transfected cells are indicated by the arrows. Bar, 15 μm.

LIMK1. LIMK activity is further regulated by homodimerization and transphosphorylation of several serines, which are required for downstream signaling, including cofilin phosphorylation (10, 32). LIMK short is also a dominant-negative protein that lacks 20 amino acids in the catalytic domain, so it does not have kinase activity but can be phosphorylated on T508 (7). Although nischarin does not bind to dominant-negative LIMK (D460N), it binds to another dominant-negative protein, LIMK short (46). In addition, our data clearly indicate that the LIMK protein bound to nischarin is phosphorylated on threonine 508, suggesting that this phosphorylation of LIMK is very important for the interaction with nischarin. Furthermore, a mutation of this threonine to valine abolished the interaction, suggesting that this initial phosphorylation is crucial for interaction. This interaction represents a novel method of nischarin-mediated regulation of LIMK.

Although it is clear that nischarin plays a key role in the regulation of LIMK activity and the control of cell invasion, the precise mechanisms are not yet fully understood. Interestingly, nischarin reduces phosphorylation of T508 upon binding. It is possible that nischarin binding to LIMK disrupts dimerization of LIMK (which is required for activation of LIMK), which in turn causes inhibition of LIMK. A similar situation has been depicted for GSK3β regulation of MEKK4 kinase function (GSK3β and MEKK4 association affects dimerization of MEKK4, which leads to inhibition of MEKK4) (1). It is known that T508 is phosphorylated by PAK and ROCK, and this phosphorylation is important for subsequent phosphorylation events on LIMK1. It is likely that association of nischarin to LIMK and inhibition of LIMK T508 phosphorylation may not allow these subsequent phosphorylation events to take place, and thus nischarin affects LIMK phosphorylation. Consistent with this idea, nischarin was able to block cofilin phosphorylation. However, the exact mechanism by which nischarin inhibits the activity of LIMK is still not yet clear. It is known that T508 is phosphorylated by PAK and ROCK, and this phosphorylation is important for full activation of LIMK1. Consistent with this idea, nischarin blocks cofilin phosphorylation. However, how the dephosphorylation occurs is not clear. It is interesting that RhoE binds to ROCK 1 and prevents ROCK1 from phosphorylating downstream effectors (44), and more interestingly, ROCK1 can also phosphorylate RhoE (45). The exact mechanisms by which these two proteins (ROCK and RhoE) regulate each other are not clearly understood. Thus, nischarin's effect on LIMK may be as complex as RhoE regulation of ROCK functions. As described above, LIMK can be activated by non-PAK-mediated signaling events (34). Our data indicate that association of nischarin with LIMK seems to be independent of PAK association with nischarin, because suppression of PAK does not have any effect on the association between LIMK and nischarin. Also, the effect of nischarin on LIMK seems to be direct and not mediated through PAK, suggesting that nischarin may have simultaneously associate with these proteins.

Cell migration is one of the hallmarks of cell invasion. Growth factor stimulation of carcinoma cells produces lamellipodia and invadopodia (56). In order to move the membrane forward, actin polymerization must occur at the plasma membrane and actin filaments must be disassembled at the rear end, so that actin monomers can be replenished for further polymerization at the leading edge (42). Several extracellular stimuli induce changes in actin organization; however, very little is known about the mechanism by which they control actin polymerization. Directional migration of carcinoma cells depends on the spatial and temporal regulation of the actin cytoskeleton by several proteins, such as cofilin, Arp2/3 complex, profilin, and WASP (38, 42, 52). Cofilin plays a pivotal role in promoting actin depolymerization at the pointed ends and severing long actin filaments at the rear end, which leads to rapid turnover of actin filaments (23). In addition, cofilin activity is essential for the formation of the free barbed ends that are required for the formation of lamellipodial protrusions (12, 19, 37). Inactivated cofilin cannot promote the actin filament depolymerization that leads to enhancement of stable actin filaments, which in turn causes reduced migration (49). In breast cancer cells, activation and inactivation of cofilin should be balanced for transient cofilin activation to occur (52).

LIMK regulates actin dynamics through cofilin, its only known downstream effector. Cofilin is phosphorylated by LIMK on serine 3, and phosphorylated cofilin is inactive. Phosphorylation of serine (3) by LIMK and Tes kinase (50, 59) results in cofilin inactivation, whereas dephosphorylation by SSH phosphatase and chronophin results in their reactivation (39). Inhibition of cofilin activity in carcinoma cells inhibits cell motility (28), and downregulation of cofilin expression reduces the assembly and stability of invadopodia (56), indicating a significant role of cofilin in cell invasion. Since nischarin regulates cofilin phosphorylation, it is likely that nischarin regulates cell invasion by affecting actin cytoskeleton. It is tempting to speculate that nischarin may inhibit phosphorylation of cofilin, leading to increased levels of active cofilin, which in turn decreases lamellipodia extension/cell migration. However, the situation is more complex, since recent work on cofilin has reversed previous suggestions that the phosphorylation and inactivation of cofilin are necessary for motility (23). The process of migration is simplistically compared with a treadmill-type reaction, with addition of subunits at the barbed end and loss of subunits at the pointed end (42).

As discussed above, cofilin plays an important role in severing function, whereas the Arp2/3 complex is involved in branching activity; both these activities together generate a propulsive force at the leading edge that stimulates protrusion (23, 42). Cofilin activity has been shown to be required for directional migration but not for lamellipodial formation per se (15, 18, 37). Nischarin siRNA induced aberrant F actin assembly, resulting in multiple long protrusions and enhanced cell invasion. The inappropriate F actin organization in Nischarin siRNA cells could be the result of LIMK1/cofilin enhancement. Dawe et al. (18) showed that overexpression of LIMK1 inactivates cofilin and induces several protrusions in random directions that resulted in problems in cell polarity and directionality of fibroblasts. Although the morphology of the cells in this study was different from what we have observed in nischarin knockdown cells, at least one possibility is that nischarin siRNA upregulation of LIMK/cofilin may cause the actin reorganization and increase in cell invasion. However, this may not be the sole mechanism, as it is known that cofilin is regulated by multiple pathways using several proteins, including LIMK1, LIMK2, NRK, TESK1, TESK2, and phosphatase types 1, 2A, and 2B, slingshot (SSH), and chronophin

phosphatases (52). Also, it is known that stimulatory and inhibitory branches of the cofilin pathways must function properly, and too much or too little activity will inhibit the essential steps in migration and invasion (52).

Sonnenberg and colleagues (15) clearly showed that α5β1-integrin-expressing cells that adhered to fibronectin increased cofilin phosphorylation (decreased activity), which led to problems in cell polarity and directional migration. Interestingly, these α5-expressing cells produced long and thin membranous protrusions; the phenotype is somewhat similar to the one induced by nischarin siRNA. Although α5β1 cells have impaired cell polarity and directional migration, the α5β1 indeed stimulated total migration. Thus, similarly, nischarin siRNA stimulates cell migration (6) and invasion in spite of some changes in membrane protrusions. It is possible that nischarin siRNA cells may have problems in directional migration as well. Coincidentally, nischarin knockdown cells were also plated on fibronectin.

Similar to our observations, neurofibromin (NF1) siRNA induced cofilin inactivation through the Rho-ROCK-LIMK pathway (40) to alter the actin cytoskeleton reorganization, promote cell motility, and invasion. It has been hypothesized that cells may acquire enhanced invasive property if the leading and rear edges of the cells are not balanced (40), and this could be another possible means by which nischarin siRNA stimulates cell invasion. Since it is known that temporal and spatial control of LIMK and cofilin are essential for directional migration and uncontrolled changes in either of the proteins may lead to uncontrolled migration and invasion (38), our findings support that the deregulation of nischarin affects tumor cell migration and invasion.

LIM kinase and cofilin are highly expressed in invasive cells (53). Several reports indicate that LIM kinase promotes cell invasion in vitro (16, 61), increases cell proliferation in vitro, increases tumor growth, and induces lung and liver metastases in vivo (8). Thus, LIMK has been suggested to be a good target for antimetastasis therapy (48). It will be interesting, therefore, to investigate how these proteins (LIMK, cofilin, and nischarin) coordinately regulate actin polymerization in invadopodia during cell invasion. Because nischarin is a novel regulator of LIMK-driven cell invasion, it will also be interesting to examine whether nischarin is also involved in tumorigenesis by regulating LIMK and cofilin.

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