Molecular Biology of the Cell
Vol. 21, 4120–4129, December 1, 2010

The Roles of Two Distinct Regions of PINCH-1 in the Regulation of Cell Attachment and Spreading

Satoko Ito, Yuko Takahara, Toshinori Hyodo, Hitoki Hasegawa, Eri Asano, Michinari Hamaguchi, and Takeshi Senga

Division of Cancer Biology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, 466-8550, Japan

Submitted May 25, 2010; Revised September 6, 2010; Accepted September 27, 2010
Monitoring Editor: Kozo Kaibuchi

Cells attach to the extracellular matrix (ECM) through integrins to form focal adhesion complexes, and this process is followed by the extension of lamellipodia to enable cell spreading. PINCH-1, an adaptor protein essential for the regulation of cell–ECM adhesion, consists of five tandem LIM domains and a small C-terminal region. PINCH-1 is known to interact with integrin-linked kinase (ILK) and Ras suppressor protein 1 (Rsu-1); however, the precise mechanism by which this complex regulates cell–ECM adhesion is not fully understood. We report here that the LIM1 domain of PINCH-1, which associates with ILK to stabilize the expression of this protein, is sufficient for cell attachment but not for cell spreading. In contrast, the C-terminal region of PINCH-1, which binds to Rsu-1, plays a pivotal role in cell spreading but not in cell attachment. We also show that PINCH-1 associates with Rsu-1 to activate Rac1 and that Rac1 activation is necessary for cell spreading. Thus, these data reveal how specific domains of PINCH-1 direct two independent pathways: one utilizing ILK to allow cell attachment, and the other recruiting Rsu-1 to activate Rac1 in order to promote cell spreading.

INTRODUCTION

Adhesion of cells to the extracellular matrix (ECM) is crucial for a variety of cellular processes such as migration and changes in cell shape. Integrins are a family of transmembrane proteins that link the ECM with intracellular signaling molecules and the actin cytoskeleton (Hynes, 1992; Schwartz *et al*., 1995; Dedhar and Hannigan, 1996). The binding of ligands to integrins triggers diverse signaling pathways and the recruitment of numerous molecules to form focal adhesions and related structures. Over the last two decades, studies have focused on identifying the components of focal adhesions, and various molecules have been found to be either stably or transiently associated with cell–ECM adhesions (Zamir and Geiger, 2001; DeMali *et al*., 2003). Elucidating the function of each of these molecules is essential to fully understand the molecular basis of the cell behaviors mediated by cell–ECM adhesions.

Recent studies from a number of laboratories have provided evidence that the cytoplasmic protein complex ILK-PINCH-parvin (IPP) is essential for the control of cell–ECM adhesion-mediated cell behavior (Wu, 2004 and 2005; Legate *et al*., 2006). The PINCH family of adaptor proteins consists of five tandem LIM domains and a small C-terminal region (Rearden, 1994; Tu *et al*., 1999). Two members of this family, PINCH-1 and PINCH-2, are widely expressed in mammalian cells and localize to sites of cell–ECM adhesion. Accumulating evidence indicates that PINCH-1 plays a pivotal role in the regulation of diverse cellular functions such as cell survival, migration, and spreading (Fukuda *et al*., 2003; Xu *et al*., 2005; Chen *et al*., 2008). PINCH proteins associate with the N terminus of ILK through the most N-terminal LIM (LIM1) domain independent of adhesion signals, and this interaction is required for the stability of both proteins (Tu *et al*., 1999; Fukuda *et al*., 2003; Chiswell *et al*., 2008). ILK, which was first identified based on its interaction with the cytoplasmic domain of a β1-integrin subunit, regulates various cell–ECM adhesion-mediated signal pathways (Hannigan *et al*., 1996). ILK comprises four N-terminal ankyrin repeats, a central pleckstrin homology (PH) domain, and a C-terminal catalytic domain (Dedhar *et al*., 1999). The catalytic domain of ILK interacts with other components of the IPP complex, including α-parvin and β-parvin. α-Parvin and β-parvin are ubiquitously expressed and consist of two C-terminal calponin homology (CH) domains referred to as CH1 and CH2 (Tu *et al*., 2001; Yamaji *et al*., 2001). Previous studies have shown that parvins are essential components of IPP that regulate cell attachment, spreading and activation of Rac (Tu *et al*., 2001; Yamaji *et al*., 2001; Zhang *et al*., 2004).

Rsu-1 was first identified as a gene that inhibits transformation by Ras (Cutler *et al*., 1992). Rsu-1 is a highly conserved protein with leucine-rich repeats (LRR) that is expressed in various mammalian cells. LRR motifs are present in a wide range of proteins and have been implicated in protein-protein interactions (Kobe and Kajava, 2001). Although expression of Rsu-1 has been reported to block the activation of Jun kinase and ROCK (Masuelli and Cutler,

This article was published online ahead of print in *MBoC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10–05–0459) on October 6, 2010.

Address correspondence to: Takeshi Senga (tsenga@med.nagoya-u.ac.jp).

1996), the physiological role of Rsu-1 remains unknown. Recent reports have demonstrated that the association of Rsu-1 with PINCH-1 regulates integrin-mediated signaling pathways (Dougherty *et al*., 2005 and 2008). In *Drosophila*, the association of PINCH-1 and Rsu-1 regulates integrin and JNK signaling pathways during embryonic development (Kadrmas *et al*., 2004).

Previously, it was demonstrated that PINCH-1 was critical for cell spreading (Xu *et al*., 2005); however, the mechanism by which PINCH-1 regulates cell spreading has not been elucidated. In this report, we further explored the role of PINCH-1 in cell spreading and found that two independent regions of PINCH-1 regulate distinct functions. We demonstrate here that the LIM1 domain is essential for cell attachment, while the C-terminal tail mediates the association of PINCH-1 with Rsu-1 and is specifically required for cell spreading. Furthermore, we show that Rac1 activation during cell spreading is regulated by the PINCH-1-Rsu-1 pathway.

MATERIALS AND METHODS

Cell Culture and Antibodies

MCF10A, a human immortalized mammary epithelial cell line, was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM-F12 medium (Invitrogen, Carlsbad, CA) supplemented with 0.1 μg/ml cholera toxin (Sigma, St. Louis, MO), 0.02 μg/ml epidermal growth factor (PeproTech, Rocky Hill, NJ), 10 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% horse serum (Invitrogen) (Debnath *et al*., 2003). 293T cells and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (Equitech BIO, Kerriville, TX) and penicillin/streptomycin. Antibodies were purchased from the following manufacturers: anti-vinculin and anti-β-actin antibodies (Sigma); anti-PINCH, anti-ILK, anti-paxillin, anti-FAK, anti-Rac1, anti-E-cadherin and anti-vimentin antibodies (BD Biosciences, San Jose, CA); anti-GFP antibody (Nacalai Tesque, Tokyo, Japan); anti-Rsu-1 antibody (Protein Tech Group, Chicago, IL).

Plasmids

Human PINCH-1 and Rsu-1 cDNAs were amplified by PCR from HeLa cDNA. Full-length PINCH-1 and PINCH-1 deletion mutants were cloned into pQCXIN retroviral vectors (Clontech, Mountain View, CA) with an N-terminal GFP tag or pcDNA3.1 vectors (Invitrogen) with an N-terminal HA tag. Full-length Rsu-1 and Rsu-1 deletion mutants were cloned into pQCXIP retroviral vectors (Clontech) with an N-terminal GFP tag or pcDNA3.1 vectors with an N-terminal Myc tag. Rac1 cDNA was kindly provided by Dr. Kaibuchi and cloned into the pQCXIH retroviral vector (Clontech). To produce recombinant proteins in bacteria, cDNAs of PINCH-1 deletion mutants were cloned into the pGEX5X-1 vector (GE Healthcare, Uppsala, Sweden). Oligonucleotides encoding shRNA specific for human PINCH-1 (5′-GACCTATGAATGGTTTTAT-3′) and luciferase (5′-CTTACGCTGAGTACTTCGA-3′) were cloned into the pSIREN-RetroQ retroviral vector (Clontech).

Generation of Stable Cell Lines

293T cells were transfected with the pSIREN-RetroQ retroviral vector encoding either control shRNA specific for luciferase or PINCH-1 shRNA in combination with the pVPack-GP and pVPack-Ampho vectors (Stratagene, Tokyo, Japan) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Culture supernatants were collected 48 h after transfection and applied to MCF10A cells in combination with 2 μg/ml polybrene (Sigma). Cells were cultured for 24 h, and infected cells were selected with 1 μg/ml puromycin. To establish cells that reexpressed wild-type PINCH-1 and PINCH-1 deletion mutants, PINCH-1 shRNA-expressing cells were infected with retroviruses that encoded these genes and selected with 400 μg/ml G418. Cells that expressed active Rac1 were produced by infecting cells with retrovirus containing active Rac1 and selected with 50 μg/ml hygromycin. MCF10A cells that expressed wild-type and deletion mutant Rsu-1 were produced by infecting cells with retroviruses and selected with 1 μg/ml puromycin.

siRNA Transfection

siRNAs specific for Rsu-1 were designed and synthesized by Sigma. The sequences of the siRNAs were 5′-GCUUGGCGUGUCCCAUGUUUU-3′ and 5′-CCUUCUCUCUCCAACACTT-3′. The sequence of control siRNA targeting luciferase (Sigma) was 5′-CUUACGCUGAGUACUUCGATT-3′. Cells were transfected with 20 nM siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after the siRNA transfection, cells were used for the indicated assays.

Immunoblotting and Immunoprecipitation

Cells were lysed with Laemmli sample buffer (20% glycerol, 135 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-Mercaptoethanol, 0.003% BPB) and boiled for 5 min. Protein concentrations of lysates were measured using the RC-DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Equal protein quantities were separated on SDS-polyacrylamide electrophoresis (SDS-PAGE) gels and transferred to PVDF membrane (Millipore, Billerica, MA). The membranes were blocked with 1% nonfat skim milk, incubated with each primary antibody for 1 h, washed with TBS-T buffer (100 mM Tris-HCl, pH 7.4, 9% NaCl, 0.5% Tween20), and incubated with secondary antibodies. Proteins were visualized by enhanced chemiluminescence (GE Healthcare). To detect associations between PINCH-1 deletion mutants and Rsu-1, HeLa cells were transfected with vectors encoding PINCH-1 deletion mutants using FuGENE (Roche, Mannheim, Germany) according to the manufacturer's protocol. Cells were lysed 24 h later with TNE buffer [25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40 (WAKO, Osaka, Japan)] and centrifuged at 15000 rpm for 20 min to clear cell debris. Cell lysates containing equal amounts of protein were incubated with anti-HA antibody coupled to protein A-agarose beads (Thermo Scientific, Waltham, MA) at 4°C for 1 h. After extensive washing with lysis buffer, proteins were eluted with Laemmli sample buffer, boiled, and then subjected to immunoblotting.

Time-Lapse Analysis

Cells were seeded onto fibronectin-coated glass base dishes (IWAKI, Tokyo, Japan) and observed using a time-lapse microscope system (IX81-ZDC, Olympus, Tokyo, Japan) with a noncooled camera (Retiga Exi FAST, Q-Imaging, Surrey, BC, Canada). Images were acquired and analyzed using MetaMorph Imaging System (Universal Imaging, Silicon Valley, CA).

Cell Attachment Assay

Cells were seeded onto a 24-well plate coated with fibronectin at a density of 1×10^5 cells per well. After 20 min, unattached cells were removed by tapping the plate and rinsing the wells with PBS twice. Attached cells were counted in five representative high-power fields. Results represent the relative ratio of the number of attached cells in each experimental group to that in control shRNA-expressing cells. The data are presented as the average of the results from three independent experiments.

Cell Spreading Assay

Cells were seeded onto a 24-well plate coated with fibronectin at a density of 1×10^5 cells per well and fixed 1 h later. Spread and nonspread cells were counted in five representative high-power fields. Nonspread cells were defined as small round cells with few or no membrane protrusions, whereas spread cells were defined as large cells with extensive visible lamellipodia. Results represent the percentage of spread cells in five high-power fields. The data are presented as the average of the results from three independent experiments.

Protein Identification by Mass Spectrometry

An HA-PINCH-1-expressing 293T cell line was generated by infecting cells with retrovirus. Cells were lysed with TNE buffer, and cell lysates were applied to disposable plastic columns (Thermo Scientific) packed with anti-HA affinity matrix (Roche). After washing the columns with TNE buffer, the matrix was transferred to the tube and suspended in Laemmli sample buffer. Samples were separated on 10% SDS-PAGE gels and stained using a SilverQuest Silver Staining Kit (Invitrogen). Protein bands were excised and cut into 1-mm^3 pieces. Gel pieces were destained, reduced, alkylated, and digested using an In-Gel Tryptic Digestion Kit (Thermo Scientific) according to the manufacturer's protocol. Peptides were sequenced using the LC-MS/MS system (MAGIC2002, Michrom Bioresources, Sacramento, CA; HTC-PAL, CTC Analytics AG, Zwingen, Swiss; LCQ Advantage, Thermo Scientific), and proteins were identified using the Mascot software package (Matrix Science, London, UK).

GST Pull-Down Assay

To detect interactions between PINCH-1 and Rsu-1, Myc-tagged Rsu-1 was transiently expressed in 293T cells, which were lysed with pull-down buffer [5% glycerol, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM $MgCl_2$, protease inhibitor cocktail (Roche), 1 mM PMSF]. Cell lysates were incubated with GST-fused LIM5 or LIM5+C bound to glutathione agarose beads (Sigma) for 1 h at 4°C. After washing the beads with pull-down buffer four times, proteins were eluted with Laemmli sample buffer, boiled, and then subjected to immunoblotting.

Rac Activity Assay

Cells were lysed with pull-down lysis buffer and incubated with GST-PAK-PBD (residues 67-150) fusion protein bound to glutathione-agarose beads for

1 h at 4°C. Beads were washed with pull-down buffer four times and then subjected to immunoblotting with anti-Rac antibody to detect active Rac protein bound to GST-PAK-PBD. Total Rac protein was detected by immunoblotting of cell lysates.

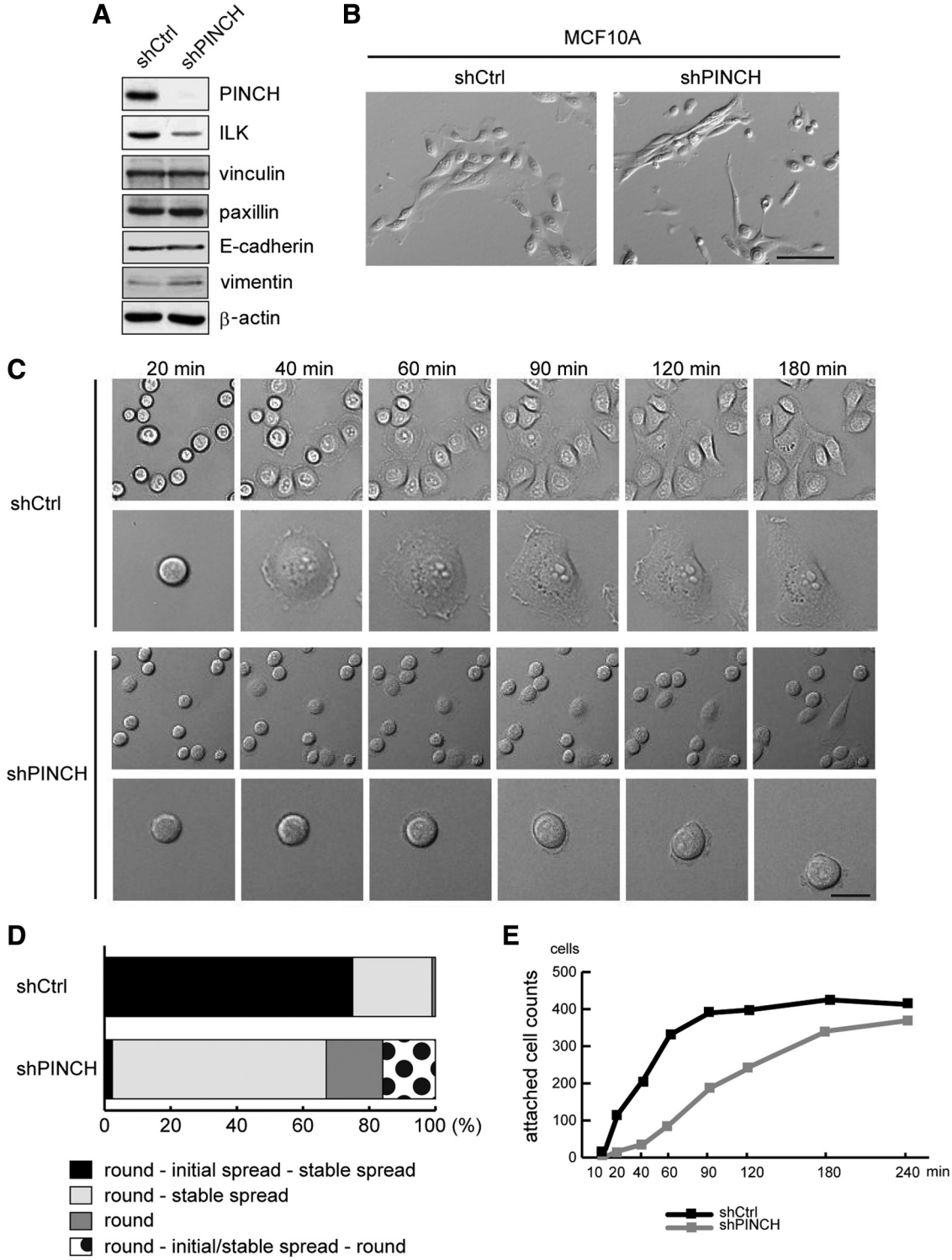

Figure 1. PINCH-1 is required for attachment and spreading of MCF10A cells. (A) Expression of PINCH-1, ILK, vinculin, paxillin, E-cadherin, and vimentin in shCtrl and shPINCH cells was examined by Western blot. (B) Morphology of shCtrl and shPINCH cells was examined using phase contrast microscopy (scale bars = 100 μm). (C) Time-lapse analysis of shCtrl and shPINCH cells after seeding onto fibronectin-coated dishes (scale bar = 20 μm). (D) Cell spreading of shCtrl and shPINCH cells was analyzed using time-lapse microscopy for 8 h after seeding onto fibronectin-coated dishes. Cells were classified based on their process of spreading. (shCtrl, n = 191; shPINCH, n = 210) (E) Cell attachment assays of shCtrl and shPINCH cells. Cells (1×10^5) were seeded onto fibronectin-coated 24-well plates, unattached cells were washed out, and attached cells in three random microscopic fields were counted at the indicated times.

Statistical Analysis

Data are expressed as mean ± SD. Comparisons between the groups were performed using unpaired *t* tests. *P* values of <0.05 were considered statistically significant.

RESULTS

When cells are seeded onto surfaces coated with ECM, they initially attach to the ECM through integrins and form focal complexes along the periphery of cells. These integrin–ECM interactions initiate robust early membrane extensions, after which cells retract membrane protrusions, resulting in a stable shape (Price *et al*., 1998, Zhang *et al*., 2008). To investigate the function of PINCH-1 during the process of cell spreading, we used RNA interference to down-regulate PINCH-1 expression in MCF10A cells. In comparison with control shRNA (shCtrl)-expressing MCF10A cells, PINCH-1 expression was significantly suppressed in MCF10A cells that expressed shRNA targeting the 3′-UTR of PINCH-1 (shPINCH) (Figure 1A). Consistent with previous results (Fukuda *et al*., 2003), suppression of PINCH-1 expression resulted in a reduction in the expression of ILK, whereas expression of vinculin and paxillin was not affected (Figure 1A). MCF10A cells are nontransformed mammary epithelial cells with well-organized cell–cell junctions. Similarly, shCtrl cells showed epithelial morphology with well-organized cell junctions. In contrast, shPINCH cells displayed a mesenchymal/fibroblastic morphology with decreased cell–cell junctions, and some shPINCH cells remained rounded without membrane extensions (Figure 1B). We examined whether epithelial to mesenchymal transition (EMT) was induced by PINCH-1 knockdown, but the expressions of E-cadherin and vimentin were similar between shCtrl and shPINCH cells (Figure 1A). In addition, there was no difference in the rate of cell proliferation between these cell lines,

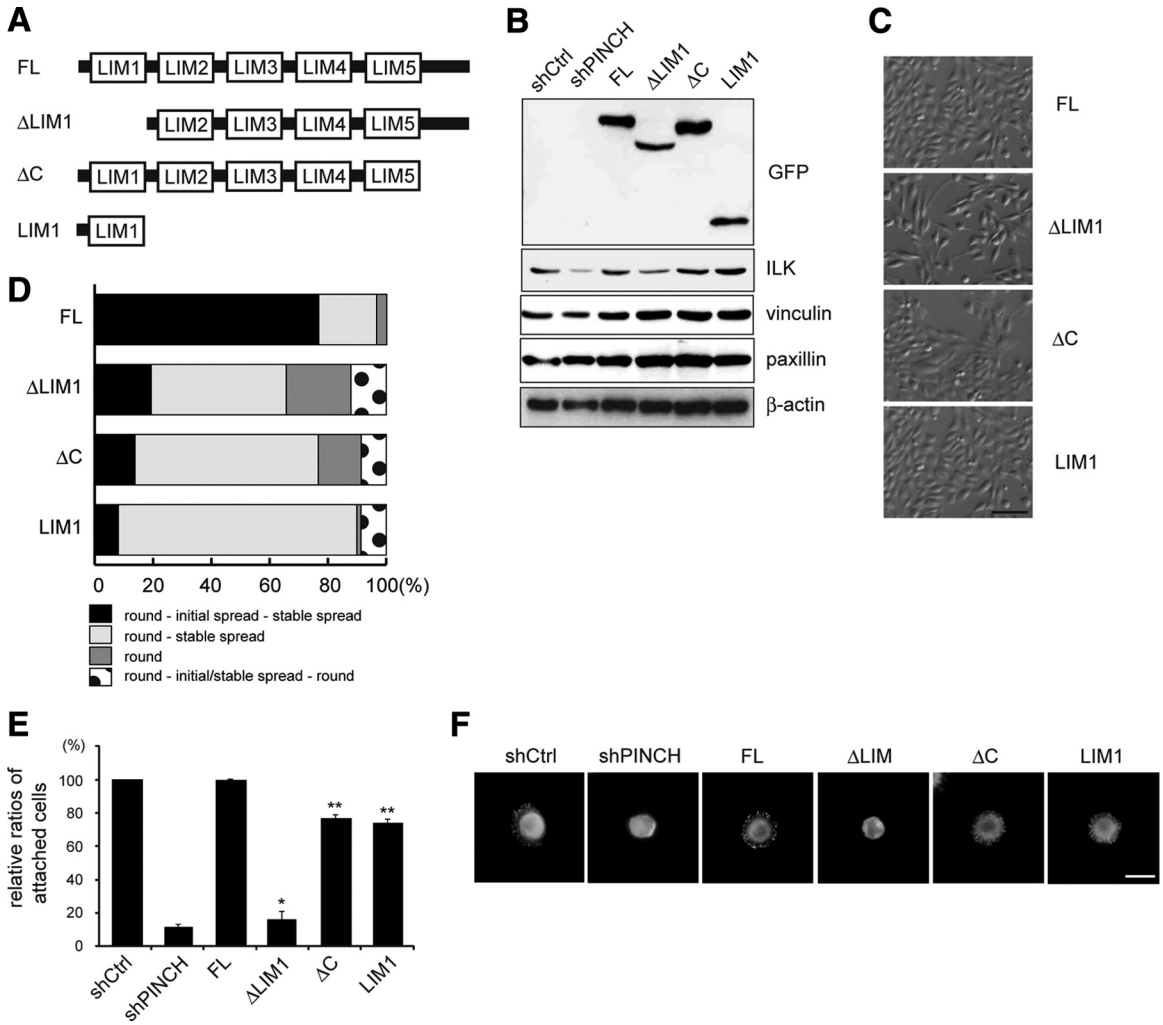

Figure 2. Different roles of LIM1 domain and C-terminal region of PINCH-1 in cell attachment and spreading. (A) Schematic representation of PINCH-1 deletion mutants that were used in the experiments: FL, aa1–325; ΔLIM1, aa63–325; ΔC, aa1–304; LIM1, aa1–69. (B) Expression of PINCH-1 deletion mutants and focal adhesion proteins was examined by Western blot. (C) Cell morphology was examined using phase contrast microscopy (scale bar = 100 μm). (D) shCtrl, shPINCH, FL, ΔLIM1, ΔC, and LIM1 cells were seeded onto fibronectin-coated dishes, and cell spreading was analyzed using time-lapse microscopy for 8 h. (FL, n = 121; ΔLIM1, n = 132; ΔC, n = 127; LM1, n = 129) (E) Cell attachment assays of shCtrl, shPINCH, FL, ΔLIM1, ΔC, and LIM1 cells. Percentages of attached cells relative to shCtrl cells are represented as a graph. Data represent means ± SD from three independent experiments. Asterisks indicate P values in comparison with shPINCH (*$p > 0.05$, **$p < 0.001$). (F) shCtrl, shPINCH, FL, ΔLIM1, ΔC and LIM1 cells were seeded onto fibronectin-coated coverslips, fixed 20 min later, and immunostained with anti-vinculin antibody (scale bar = 20 μm).

and apoptotic cells were not observed among shPINCH cells (data not shown), indicating that PINCH-1 silencing in MCF10A cells did not affect cell viability.

To investigate the effect of PINCH-1 silencing on cell spreading, we observed shCtrl and shPINCH cells using time-lapse microscopy. On attachment to the fibronectin-coated surface, shCtrl cells began to produce extensive membrane protrusions. After this initial spreading, cells retracted their protrusions and took on a stably spread morphology (Figure 1C). Similar to previous reports, spreading of shPINCH cells was significantly delayed (Fukuda *et al*., 2003). In addition to this delay in spreading, we observed a marked difference in the process of cell spreading between shCtrl and shPINCH cells. A number of shPINCH cells produced small protrusions after attachment and gradually spread to take on an elongated shape without going through the stage of initial spreading (Figure 1C). As shown in Figure 1D, observation of each cell by time-lapse microscopy for 8 h revealed that more than 70% of shCtrl cells demonstrated extensive membrane protrusions and then retracted these protrusions. In contrast, <3% of shPINCH cells showed robust membrane protrusions, and nearly 60% of these cells gradually spread without showing any signs of initial spreading. Interestingly, ~15% of shPINCH cells became round again after spreading, which is consistent with a previous report by Stanch *et al.* that PINCH-1$^{-/-}$ cells displayed frequent cell rounding because of reduced cell–ECM contacts (Stanchi *et al*., 2009). We also performed cell attachment assays to examine the ability of each cell line to attach to the fibronectin-coated surface. As shown in Figure 1E, cell attachment was significantly delayed in shPINCH cells compared with that in shCtrl cells. These results indicate that PINCH-1 is essential for cell spreading and cell attachment.

PINCH-1 consists of five tandem LIM domains and a short C-terminal region. To determine which region was critical for cell attachment and cell spreading, we infected shPINCH cells with recombinant retrovirus that encoded GFP-tagged PINCH-1 deletion mutants and established stable cell lines. LIM1 contains the most N-terminal LIM domain, and ΔLIM1 and ΔC are deleted of the LIM1 domain and the small C-terminal region, respectively (Figure 2, A and B). ΔLIM1 cells showed reduced expression of ILK, but ΔC and LIM1 cells maintained ILK expression at levels similar to those in shCtrl and FL cells (Figure 2B). These results are consistent with the previous finding that the association of ILK with the LIM1 domain of PINCH-1 is essential for the stabilization of PINCH-1 (Stanchi *et al*., 2009). The expression of FL, ΔC, or LIM1 in shPINCH cells restored the epithelial morphology of MCF10A cells, whereas ΔLIM1 cells displayed a mesenchymal/fibroblastic morphology similar to that of shPINCH cells (Figure 2C). To examine spreading in each cell line, cells were trypsinized, reseeded onto fibronectin-coated dishes, and monitored for 8 h by time-lapse microscopy. As shown in Figure 2D, initial spreading, which is defined by a robust outward extension of the cell membrane upon attachment, was observed in more than 70% of FL cells. In contrast, initial cell spreading was significantly suppressed in ΔLIM1, ΔC, and LIM1 cells. We next examined cell attachment in these cell lines. As shown in Figure 2E, cell attachment assays showed that the defects observed in shPINCH cells were rescued in ΔC and LIM1 cells but not in ΔLIM1 cells. We immunostained each cell line for vinculin to observe the formation of focal complexes 20 min after reseeding cells on the fibronectin-coated surface. Consistent with the results obtained from cell attachment assays, shCtrl, FL, ΔC, and LIM1 cells formed focal complexes along the periphery of cells, whereas focal complex formation was suppressed in shPINCH and ΔLIM1 cells. These results indicate that the interaction of PINCH-1 with ILK is crucial for cell attachment and cell spreading. Furthermore, these findings demonstrate that the LIM1 domain is sufficient for cell attachment, but not for cell spreading, and that the small C-terminal region is required for cell spreading.

Rac1, a small GTPase that regulates lamellipodia formation, is known to be activated upon cell adhesion to the ECM to induce early membrane protrusions (Sepulveda *et al*., 2005). We measured active Rac1 levels during cell spreading using a pull-down assay, which utilizes the interaction of active Rac1 and GST-PAK-PBD. As previously reported, silencing of PINCH-1 expression suppressed the activation of Rac1 (Zhang *et al*., 2004) (Figure 3A). Interestingly, every mutant cell line also showed reduced activation of Rac1 during cell spreading (Figure 3B). Because Rac1 is a critical regulator of cell spreading, we examined whether expression of active Rac1 could overcome the spreading defect observed in mutant cells. Each mutant cell line was infected with the retrovirus that encoded active Rac1 (Rac1 V12), and cell spreading was assessed. Trypsinized cells were seeded onto fibronectin-coated dishes and fixed 60 min later. This time point was chosen because initial cell spreading was most often observed 60 min after seeding. As shown in Figure 3C, expression of active Rac1 restored cell spreading in ΔC and LIM1 cells; however, shPINCH and ΔLIM1 cells were completely resistant to active Rac1-mediated cell spreading.

These results indicate that the C-terminal region of PINCH-1 specifically regulates cell spreading and Rac1 activation. To identify the signaling pathway in which the

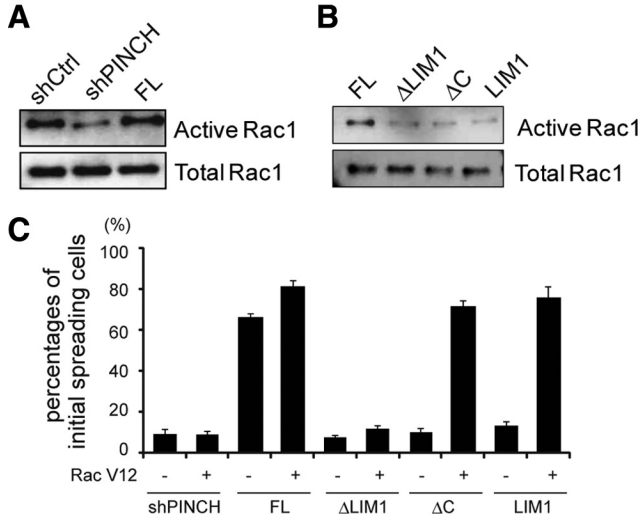

Figure 3. Expression of active Rac1 overcomes spreading defects in ΔC and LIM1 cells but not in shPINCH or ΔLIM1 cells. (A) Rac1 activity of shCtrl, shPINCH, and FL cells. Cells were seeded onto fibronectin-coated dishes and lysed 1 h later to examine Rac1 activity. Upper panel: active Rac1. Lower panel: total Rac1. (B) Rac1 activity of FL, ΔLIM1, ΔC, and LIM1cells. Cells were seeded onto fibronectin-coated dishes and lysed 1 h later to examine Rac1 activity. (C) shPINCH, FL, ΔLIM1, ΔC, and LIM1 cells were infected with retrovirus that contained active Rac1 (Rac V12), and stable cell lines were established. Cells were seeded onto fibronectin-coated dishes and fixed 1 h later to examine cell spreading. Three independent experiments were performed, and percentages of spread cells were quantified. Data represent means ± SD.

C-terminal region of PINCH-1 is involved, we aimed to identify proteins that interact with PINCH-1. N-terminally HA-tagged PINCH-1 was expressed in 293T cells by retroviral infection, immunoprecipitated with anti-HA-beads, and separated by SDS-PAGE. After the gel was silver stained, specific bands were excised and identified using mass spectrometry. In addition to ILK and parvin, which are components of the IPP complex, Rsu-1 was shown to be clearly associated with PINCH-1 (Figure 4A). Previous studies have demonstrated that Rsu-1 associates with the LIM5 domain of PINCH-1 (Dougherty *et al*., 2005). We tested the association of each mutant PINCH-1 protein with Rsu-1. 293T cells were transfected with each HA-tagged mutant, and cell lysates were immunoprecipitated with anti-HA antibody and probed for Rsu-1. The ΔLIM1 mutant protein associated with Rsu-1; however, the ΔC and LIM1 mutant proteins were not able to associate with Rsu-1 (Figure 4B), indicating that the C-terminal region was critical for the association of PINCH-1 with Rsu-1. Although it has been reported that Rsu-1 interacts with the LIM5 domain of PINCH-1 (Dougherty *et al*., 2005), we used a pull-down assay to demonstrate that both the LIM5 domain and the C-terminal region were required for this association (Figure 4C). Rsu-1 consists of seven tandem leucine-rich repeats

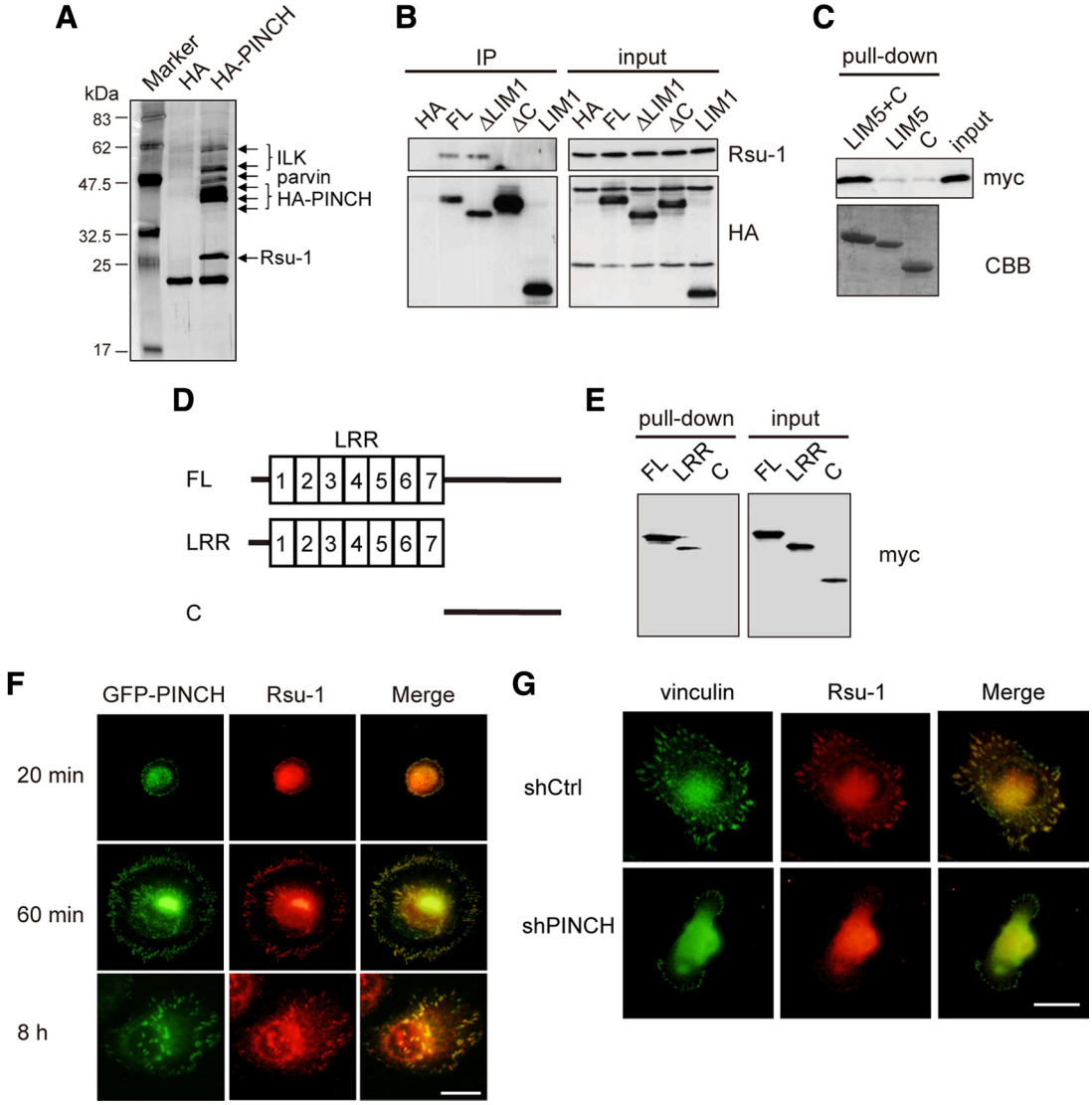

Figure 4. Association of Rsu-1 and PINCH-1. (A) 293T and HA-PINCH-1-expressing 293T cells were lysed and affinity precipitated using anti-HA affinity matrix. The precipitates were separated by SDS-PAGE and silver stained. Each band was excised and identified by LC-MS/MS. (B) 293T cells were transfected with HA-tagged FL, ΔLIM1, ΔC, and LIM1 and immunoprecipitated with anti-HA antibody. Immunoprecipitates were probed for Rsu-1 and HA. (C) 293T cells were transfected with myc-tagged Rsu-1, and cell lysates were affinity precipitated with GST and fusion proteins of GST and the indicated fragments. Precipitates were subjected to immunoblotting with anti-myc antibody. The lower panel shows Coomassie blue staining of GST fusion proteins. LIM5: aa251–304, LIM5+C: aa251–325, C: aa305–325. (D) Schematic representation of Rsu-1 deletion mutants that were used in the experiments: FL, aa1–277; LRR, aa1–202; C, aa203–277. (E) 293T cells were transfected with myc-tagged full-length or mutant Rsu-1, and cell lysates were affinity precipitated with a fusion protein of GST and LIM5+C. Precipitates were subjected to immunoblotting with anti-myc antibody. (F) FL cells were seeded onto fibronectin-coated coverslips and fixed 20 min, 60 min, or 8 h later to visualize the localization of GFP-PINCH-1 and Rsu-1 during cell spreading (scale bar = 20 μm): 20 min, attached and round; 60 min, initial spread; 8 h, stable spread. (G) shCtrl and shPINCH cells were immunostained with anti-vinculin and anti–Rsu-1 antibodies (scale bar = 20 μm).

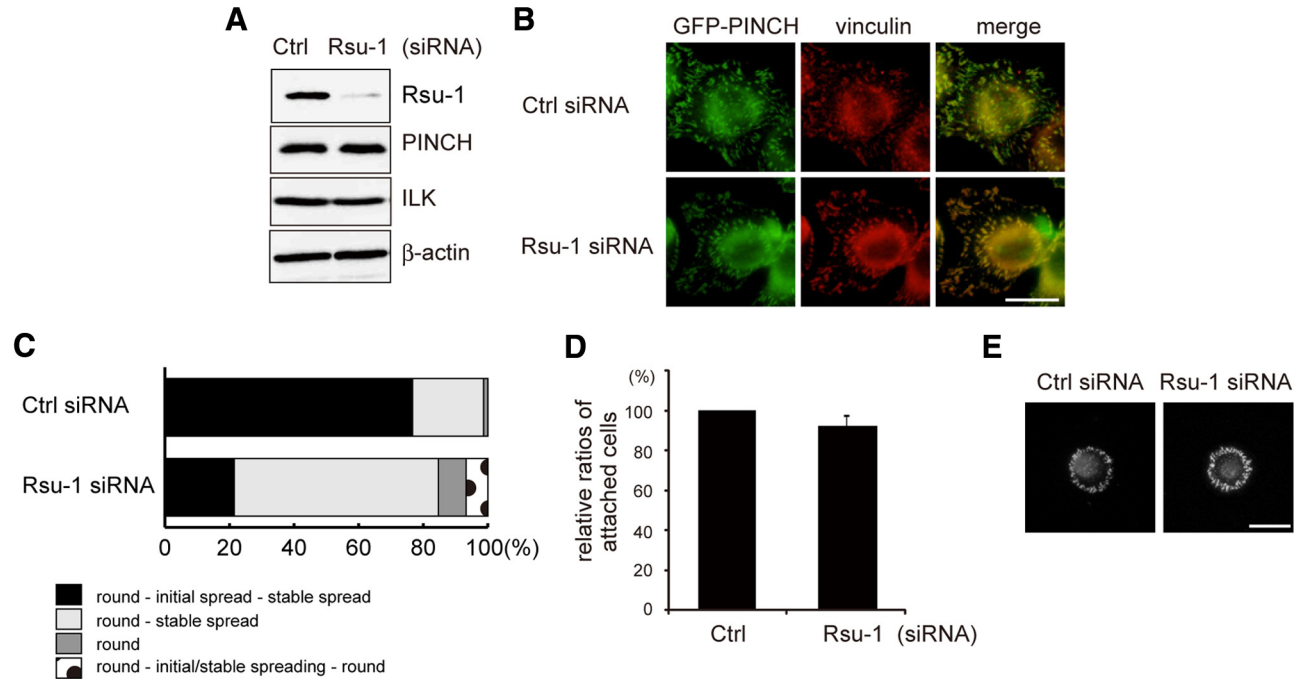

Figure 5. Rsu-1 is essential for cell spreading but not for cell attachment. (A) MCF10A cells were transfected with control or Rsu-1 siRNA, and 48 h later, expression of Rsu-1, PINCH, and ILK was examined by immunoblotting. β-actin was used as a loading control. (B) Control or Rsu-1 siRNA-transfected FL cells were fixed and immunostained with anti-vinculin antibody (Scale bar = 20 μm). (C) Control or Rsu-1 siRNA-transfected MCF10A cells were seeded onto fibronectin-coated dishes, and cell spreading was analyzed using time-lapse microscopy for 8 h. (Ctrl siRNA, n = 140; Rsu-1 siRNA, n = 147) (D) Cell attachment assays of MCF10A cells transfected with either control siRNA or Rsu-1 siRNA. Three independent experiments were performed, and the relative ratio of attached cells is indicated. (E) Control siRNA- or Rsu-1 siRNA-transfected MCF10A cells were seeded onto fibronectin-coated coverslips and fixed 20 min later. Cells were immunostained with anti-vinculin antibody (Scale bar = 20 μm).

(LRR) and a C-terminal region. We examined which region of Rsu-1 was required for the binding to LIM5 domain and C-terminal region of PINCH-1. Consistent with the previous report (Dougherty *et al*., 2005), LRR motifs were sufficient for the association with PINCH-1 (Figure 4, D and E). We next examined the localization of PINCH-1 and Rsu-1 during cell spreading. As shown in Figure 4F, Rsu-1 was colocalized with PINCH-1 at the sites of focal contact during cell spreading. We thus tested whether the localization of Rsu-1 to focal adhesions was dependent on PINCH-1. As shown in Figure 4G, Rsu-1 colocalized with vinculin at focal adhesions in shCtrl cells but not in shPINCH cells, indicating that the association with PINCH-1 is required for the proper localization of Rsu-1.

Because deletion of the PINCH-1 C-terminal region disrupted the association of PINCH-1 with Rsu-1, we hypothesized that the PINCH-1-Rsu-1 pathway was critical for cell spreading but not for cell attachment. Transfection with siRNA targeting Rsu-1 significantly reduced the expression of Rsu-1 but did not affect the expression of PINCH-1 or ILK (Figure 5A). Although silencing of PINCH-1 inhibited the localization of Rsu-1 to focal adhesions, Rsu-1 was dispensable for the localization of PINCH-1 to focal adhesions (Figure 5B). We next examined cell spreading in the absence of Rsu-1 expression. Control or Rsu-1 siRNA-transfected cells were reseeded onto fibronectin-coated surfaces and monitored for 8 h. As shown in Figure 5C, nearly 80% of control siRNA-transfected cells showed initial spreading and then retracted their membrane protrusions, taking on a stably spread morphology. In contrast, only 20% of Rsu-1 siRNA-transfected cells showed initial spreading, and nearly 60% of these cells gradually spread with small outward membrane extensions. We next tested whether Rsu-1 was essential for cell attachment. As shown in Figure 5D, cell attachment assays showed no difference between control and Rsu-1

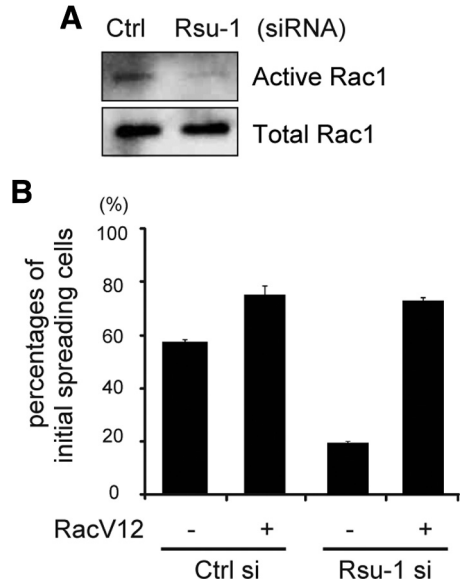

Figure 6. Rsu-1 is essential for the activation of Rac1 during cell spreading. (A) Cells transfected with control siRNA or Rsu-1 siRNA were seeded onto fibronectin-coated dishes and lysed 1 h later to examine Rac1 activity. (B) MCF10A cells that constitutively expressed active Rac1 or control vector were treated with control siRNA or Rsu-1 siRNA. Forty-eight hours posttransfection, cells were seeded onto fibronectin-coated dishes and fixed 1 h later to examine cell spreading. Three independent experiments were performed, and the ratio of spread cells is indicated.

siRNA-transfected cells. Consistent with this result, immunofluorescence analysis showed that most control and Rsu-1 siRNA-transfected cells formed focal complexes upon cell attachment (Figure 5E). These results indicate that Rsu-1 is specifically required for cell spreading but not for cell attachment.

We next examined whether Rsu-1 was required for the activation of Rac1 during cell spreading. Consistent with the suppression of cell spreading observed in cells in which Rsu-1 was silenced, activation of Rac1 was clearly reduced in Rsu-1 siRNA-transfected cells during cell spreading (Figure 6A). We tested whether active Rac1 could overcome the reduced cell spreading observed upon Rsu-1 silencing. MCF10A cells that expressed constitutively active Rac1 were transfected with control and Rsu-1 siRNA, and cell spreading was assessed. Consistent with the results obtained in ΔC cells expressing active Rac1, the expression of active Rac1 was sufficient to restore initial cell spreading in Rsu-1–silenced cells (Figure 6B). These results indicate that the PINCH-1–Rsu-1 pathway is specifically involved in Rac1 activation and cell spreading.

Lastly, we determined whether LRR motifs of Rsu-1 were sufficient for initial cell spreading. We established MCF10A cell lines that constitutively expressed either full-length or LRR motifs of Rsu-1 with N-terminal GFP tag, which were designated as FL or LRR cells, respectively. Transfection of siRNA targeting 3′-UTR of Rsu-1 suppressed expression of endogenous Rsu-1, but not of exogenously expressed proteins (Figure 7A). Both cell lines were transfected with the siRNA, and 48 h later cell spreading was monitored using time-lapse microscopy for 8 h. Initial cell spreading was observed in more than 70% of FL cells, whereas only 20% of LRR cells showed initial spreading, which is similar to the results obtained from Rsu-1 siRNA-transfected MCF10A cells in Figure 5C (Figure 7B). We then investigated Rac1 activity of both cell lines during cell spreading. Consistent with the results from cell spreading assay, activation of Rac1 of LRR cells was reduced compared with that of FL cells (Figure 7C). These results indicate that although LRR domains are sufficient for the association with PINCH-1, the C-terminal region is required for cell spreading and Rac1 activation.

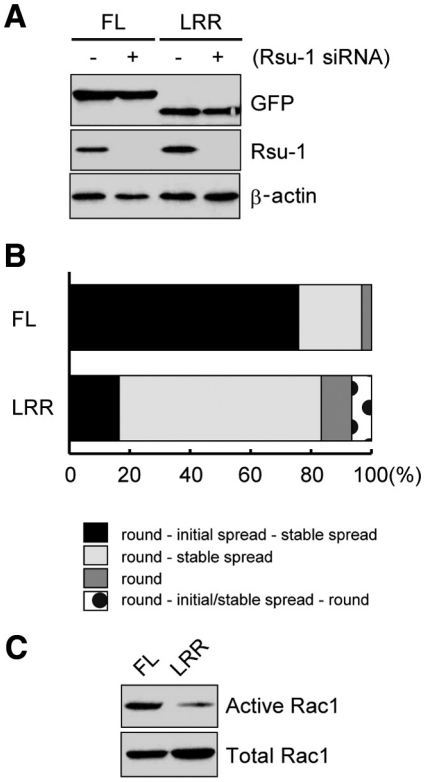

Figure 7. LRR domains of Rsu-1 is not sufficient for cell spreading and the activation of Rac1. (A) MCF10A cells that constitutively expressed either full-length or LRR domains of Rsu-1 were transfected with siRNA that targeted 3′-UTR of Rsu-1 (3′Rsu-1 siRNA). Two days later, cells were lysed and expressions of endogenous Rsu-1 and exogenously expressed Rsu-1 were examined by immunoblotting. (B) FL and LRR cells were transfected with 3′Rsu-1 siRNA and 48 h later, cells were trypsinized and seeded onto the fibronetin-coated dishes. Cell spreading was analyzed using time-lapse microscopy for 8 h. (FL, n = 92; LRR, n = 105) (C) Cells transfected with 3′Rsu-1 siRNA were seeded onto fibronectin-coated dishes and lysed 1 h later to examine Rac1 activity.

DISCUSSION

Recent studies have demonstrated that PINCH-1, an adaptor protein consisting of five tandem LIM domains and a short C-terminal region, is an essential focal adhesion molecule with multiple functions (Wu, 2004). A previous study by Xu *et al.* clearly demonstrated that PINCH-1 was required for prompt cell spreading (Xu *et al.*, 2005). In addition, the authors created deletion constructs and showed the importance of the C-terminal region for cell spreading. Despite these findings, the authors did not demonstrate which process of cell spreading was regulated by PINCH-1, and the reason that the short C-terminal region was required for cell spreading remained unclear. In this report, we demonstrated that PINCH-1 is essential not only for cell spreading but also for cell attachment. Using PINCH-1 deletion constructs, we found that the association of PINCH-1 with ILK

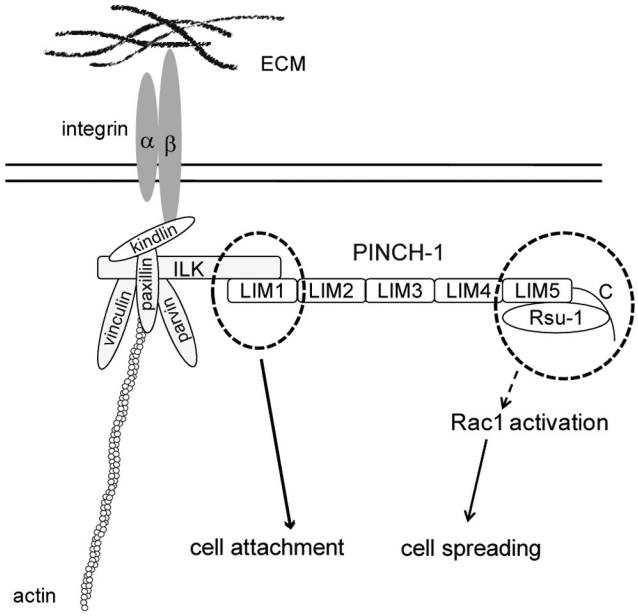

Figure 8. Proposed model for a novel role of PINCH-1 in cell attachment and cell spreading. The binding of the N terminus of ILK to the LIM1 domain of PINCH-1 is sufficient for cell attachment, whereas the association of Rsu-1 with PINCH-1 is required for Rac1-mediated cell spreading.

is essential for cell attachment and cell spreading. In addition, we showed that the LIM1 domain was sufficient for cell attachment but not for cell spreading. Furthermore, we found that the small C-terminal region of PINCH-1 was required for cell spreading because this region mediated the interaction of PINCH-1 with Rsu-1, which is essential for the activation of Rac1 during cell spreading (Figure 8).

Silencing of PINCH-1 induced morphological changes of MCF10A cells. shPINCH cells showed mesenchymal morphology with decreased cell–cell adhesion, which are characteristic features of epithelial to mesenchymal transition (EMT). Repression of E-cadherin expression is one of the hallmarks of EMT, but we did not observe any decrease of E-cadherin expression in shPINCH cells. In addition, expression of vimentin, which is induced by EMT, was similar between shCtrl and shPINCH cells. Furthermore, silencing of ILK in MCF10A cells also displayed the same morphological changes as shPINCH cells, but the expression of E-cadherin and vimentin was not affected (data not shown). Although suppression of ILK and PINCH-1 induced morphological changes similar to EMT, these changes appear to be caused by the reduced cell–ECM adhesion.

PINCH-1 and ILK associate tightly before their localization to focal adhesions, and this association is required for the maintenance of their protein expression levels (Fukuda *et al*., 2003). Consistent with these previous results, we observed reduced expression of ILK in shPINCH and ΔLIM1 cells but not in ΔC and LIM1 cells. Therefore, suppression of cell attachment and cell spreading in ΔLIM1 cells may result from the destabilization of ILK, which is a central component of the IPP complex. In addition to PINCH-1, ILK tightly associates with α-parvin and β-parvin (Sepulveda and Wu, 2006). The LIM1 domain was able to stabilize ILK and restore cell attachment, suggesting that the ILK-parvin pathway may be essential for cell attachment. Indeed, previous studies have shown that overexpression of the ILK-binding CH2 domain of parvins, which is considered to suppress downstream signaling pathways, significantly reduced cell attachment and localization of other focal adhesion components, including FAK and vinculin (Tu *et al*., 2001); however, because ILK also associates with other components of focal adhesions, including paxillin (Nikolopoulos and Turner, 2001), other pathways may also be essential for cell attachment.

In contrast to shPINCH and ΔLIM1 cells, ΔC and LIM1 cells showed recovery of cell attachment; however, cell spreading defects were similar in shPINCH, ΔLIM1 ΔC, and LIM1 cells. Immunofluorescence analysis of ΔC and LIM1 cells during the initial phase of cell spreading showed focal complex formation, which was significantly reduced in ΔLIM1 and shPINCH cells. These results indicated that the C-terminal region was specifically required for cell spreading but not for cell attachment. We thus aimed to identify proteins that associated with PINCH-1 to regulate cell spreading. Analysis of PINCH-1–interacting proteins indicated that Rsu-1 was tightly associated with the IPP complex. Previous reports have demonstrated that Rsu-1 associates with the LIM5 domain of PINCH-1 (Kadrmas *et al*., 2004; Dougherty *et al*., 2005); however, these studies used a sequence including both the LIM5 domain and the C-terminal region. We investigated the association of Rsu-1 and ΔC and found that the C-terminal region was required for the interaction between Rsu-1 and PINCH-1. Neither the LIM5 domain nor the C-terminal region alone can bind to Rsu-1; therefore, both regions appear to be necessary for this association. PINCH-2 is 82% identical to PINCH-1 at the amino acid sequence level but differs in sequence in the C-terminal region (Zhang *et al*., 2002). Previous reports have demonstrated that PINCH-2 was not able to interact with Rsu-1 (Dougherty *et al*., 2005), and overexpression of PINCH-2 suppressed cell spreading, possibly by displacing PINCH-1 (Zhang *et al*., 2002). Furthermore, the mutant PINCH-1, in which the C-terminal region has been replaced with the C terminus of PINCH-2, was not able to restore the delayed cell spreading observed in PINCH-1-silenced cells (Zhang *et al*., 2002). Together with these previous results, our findings strongly suggest that the PINCH-1-Rsu-1 interaction is crucial in regulating cell spreading.

Rac1 is a critical regulator of cell spreading (Price *et al*., 1998). Activation of Rac1 was suppressed in PINCH-1 mutant-expressing cell lines and Rsu-1–silenced cells during cell spreading. To investigate whether reduced activation of Rac1 was responsible for the suppression of cell spreading, we expressed active Rac1 and examined cell spreading. Expression of active Rac1 was not able to rescue the defect in cell spreading in shPINCH and ΔLIM1 cells. In support of these results, a previous study showed that silencing of ILK delayed cell spreading in cells that expressed active Rac1 (Boulter *et al*., 2006). Together, these results indicate that the IPP complex regulates multiple pathways in addition to Rac1 activation that are required for the regulation of cell attachment and cell spreading. Interestingly, the defect in cell spreading observed in ΔC and Rsu-1–silenced cells was fully rescued by active Rac1, indicating that the PINCH-1–Rsu-1 pathway specifically regulates Rac1 activation during cell spreading.

Rsu-1 comprises seven tandem LRR motifs and the C-terminal region. Consistent with the previous finding (Dougherty *et al*., 2005), LRR motifs of Rsu-1 were sufficient for the association with PINCH-1. Interestingly, LRR motifs were not sufficient to sustain initial cell spreading and Rac1 activation. These results indicate that the C-terminal region may mediate the association with additional molecules to regulate Rac1 activation to promote cell spreading.

Taken together, our data indicate that the LIM1 domain of PINCH-1 is sufficient for cell attachment, and the C-terminal region regulates Rac1 activation through Rsu-1 and is specifically required for cell spreading. β-parvin is known to associate with α-Pix, a guanine exchange factor for Rac1 (Rosenberger *et al*., 2003); however, there have been no studies of proteins that associate with Rsu-1 to regulate Rac1 activity. Further studies concerning the PINCH-1–Rsu-1 pathway may elucidate the function of the IPP complex, which is essential in the regulation of multiple signaling pathways that play a role in the formation of cell–ECM contacts.

ACKNOWLEDGMENTS

We thank Dr. Gary Goldberg, Molecular Biology, University of Medicine and Dentistry of New Jersey, for helpful discussions and Y. Yamakawa, Division of Medical Research Engineering, Nagoya University Graduate School of Medicine, for LC-MS/MS technical support.

REFERENCES

Boulter, E., Grall, D., Cagnol, S., and Van Obberghen-Schilling, E. (2006). Regulation of cell-matrix adhesion dynamics and Rac-1 by integrin linked kinase. FASEB J. *20*, 640–651.

Chen, K., Tu, Y., Zhang, Y., Blair, H. C., Zhang, L., and Wu, C. (2008). PINCH-1 regulates the ERK-Bim pathway and contributes to apotosis resistance in cancer cells. J. Biol. Chem. *283*, 2508–2517.

Chiswell, B. P., Zhang, R., Murphy, J. W., Boggon, T. J., and Calderwood, D. A. (2008). The structural basis of integrin-linked kinase-PINCH interactions. Proc. Natl. Acad. Sci. USA *105*, 20677–20682.

Cutler, M. L., Bassin, R. H., Zanoni, L., and Talbot, N. (1992). Isolation of *rsp-1*, a novel cDNA capable suppressing v-Ras transformation. Mol. Cell. Biol. *12*, 3750–3756.

Debnath, J., Muthuswamy, S. K., and Brugge, J. S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods *30*, 256–268.

Dedhar, S., and Hannigan, G. E. (1996). Integrin cytoplasmic interactions and bidirectional transmembrane signaling. Curr. Opin. Cell Biol. *8*, 657–669.

Dedhar, S., Williams, B., and Hannigan, G. (1999). Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signaling. Trends Cell Biol. *9*, 319–323.

DeMali, K. A., Wennerberg, K., and Burridge, K. (2003). Integrin signaling to the actin cytoskeleton. Curr. Opin. Cell Biol. *15*, 572–582.

Dougherty, G. W., Chopp, T., Qi, S., and Cutler, M. L. (2005). The Ras suppressor Rsu-1 binds to the LIM 5 domain of the adaptor protein PINCH1 and participates in adhesion-related functions. Exp. Cell Res. *306*, 168–179.

Dougherty, G. W., Jose, C., Gimona, M., and Cutler, M. L. (2008). The Rsu-1-PINCH1-ILK complex is regulated by Ras activation in tumor cells. Eur. J. Cell Biol. *87*, 721–734.

Fukuda, T., Chen, K., Shi, X., and Wu, C. (2003). PINCH-1 is and obligate partner of integrin-linked kinase (ILK) functioning in cell shape modulation, motility, and survival. J. Biol. Chem. *278*, 51324–51333.

Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar S. (1996) Regulation of cell adhesion and anchorage-dependent growth by a new β1-integrin-linked protein kinase. Nature *379*, 91–96.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Kadrmas, J. L., Smith, M. A., Clark, K. A., Pronovost, S. M., Muster, N., Yates, J. R., and Beckerle, M. C. (2004). The integrin effector PINCH regulates JNK activity and epithelial migration in concert with Ras suppressor 1. J. Cell Biol. *167*, 1019–1024.

Kobe, B., and Kajava, A. V. (2001). The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. *11*, 725–732.

Legate, K. R., Montanez, E., Kudlacek, O., and Fassler, R. (2006). ILK, PINCH and parvin: the tIPP of integrin signaling. Nat. Rev. Mol. Cell. Biol. *7*, 20–31.

Masuelli, L., and Cutler, M. L. (1996). Increased expression of the Ras suppressor Rsu-1 enhances Erk-2 activation and inhibits Jun kinase activation. Mol. Cell. Biol. *16*, 5466–5476.

Nikolopoulos, S. N., and Turner, C. E. (2001). Integrin-linked kinase (ILK) binding to paxillin LD1 motif regulates ILK localization to focal adhesions. J. Biol. Chem. *276*, 23499–23505.

Price, L. S., Leng, J., Schwartz, M. A., and Bokoch, G. M. (1998). Activation of Rac and Cdc42 by integrins mediates cell spreading. Mol. Biol. Cell *9*, 1863–1871.

Rearden, A. (1994). A new LIM protein containing an autoepitope homologous to "senescent cell antigen". Biochem. Biophys. Res. Commun. *201*, 1124–1131.

Rosenberger, G., Jantke, I., Gal, A., and Kutsche, K. (2003). Interaction of αPIX (ARFGEF6) with β-parvin (PARVB) suggests an involvement of αPIX in integrin-mediated signaling. Hum. Mol. Genet. *12*, 155–167.

Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995). INTEGRINS: Emerging paradigms of signal transduction. Annu. Rev. Cell Dev. Biol. *11*, 549–599.

Sepulveda, J. L., Gkretsi, V., and Wu, C. (2005). Assembly and signaling of adhesion complexes. Curr. Top. Dev. Biol. *68*, 183–225.

Sepulveda, J. L., and Wu, C. (2006). The parvins. Cell Mol. Life Sci. *63*, 25–35.

Stanchi, F., Grashoff, C., Yonga, C.F.N., Grall, D., Fassler, R., and Van Obberghen-Schilling, E. (2009). Molecular dissection of the ILK-PINCH-parvin triad reveals a fundamental role for the ILK kinase domain in the late stage of focal-adhesion maturation. J. Cell Sci. *122*, 1800–1811.

Tu, Y., Li, F., Goicoechea, S., and Wu, C. (1999). The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. Mol. Biol. Cell *19*, 2425–2434.

Tu, Y., Huang, Y., Zhang, Y., Hua, Y., and Wu, C. (2001). A new focal adhesion protein that interacts with integrin-linked kinase and regulates cell adhesion and spreading. J. Cell Biol. *153*, 585–598.

Wu, C. (2004). The PINCH-ILK-parvin complexes: assembly, functions and regulation. Biochim. Biophys. Acta *1692*, 55–62.

Wu, C. (2005). PINCH, N(i)ck and the ILK: network wiring at cell-matrix adhesion. Trends Cell Biol. *15*, 460–466.

Xu, Z., Fukuda, T., Li, Y., Zha, X., Qin, J., and Wu, C. (2005). Molecular dissection of PINCH-1 reveals a mechanism of coupling and uncoupling of cell shape modulation and survival. J. Biol. Chem. *280*, 27631–27637.

Yamaji, S., Suzuki, A., Sugiyama, Y., Koide, Y., Yoshida, M., Kanamori, H., Mohri, H., Ohno, S., and Ishigatsubo, Y. (2001). A novel integrin-linked kinase-binding protein, Affixin, is involved in the early stage of cell-substrate interaction. J. Cell Biol. *153*, 1251–1264.

Zamir, E., and Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. J. Cell Sci. *114*, 3583–3590.

Zhang, X., Jiang, G., Cai, Y., Monkley, S. J., Critchley, D. R., and Sheetz, M. P. (2008). Talin depletion reveals independence of initial cell spreading from integrin activation and traction. Nat. Cell Biol. *10*, 1062–1068.

Zhang, Y., Chen, K., Guo, L., and Wu, C. (2002). Characterization of PINCH-2, a new focal adhesion protein that regulates the PINCH-1-ILK interaction, cell spreading, and migration. J. Biol. Chem. *277*, 38328–38338.

Zhang, Y., Chen, K., Tu, Y., and Wu, C. (2004). Distinct roles of two structurally closely related focal adhesion proteins, α-parvins and β-parvins, in regulation of cell morphology and survival. J. Biol. Chem. *279*, 41695–41705.