Distinct Roles of Two Structurally Closely Related Focal Adhesion Proteins, α-Parvins and β-Parvins, in Regulation of Cell Morphology and Survival*

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Proteins at cell-extracellular matrix adhesions (e.g. focal adhesions) are crucially involved in regulation of cell morphology and survival. We show here that CH-ILKBP/actopaxin/α-parvin and affixin/β-parvin (abbreviated as α- and β-parvin, respectively), two structurally closely related integrin-linked kinase (ILK)-binding focal adhesion proteins, are co-expressed in human cells. Depletion of α-parvin dramatically increased the level of β-parvin, suggesting that β-parvin is negatively regulated by α-parvin in human cells. Loss of PINCH-1 or ILK, to which α- and β-parvin bind, significantly reduced the activation of Rac, a key signaling event that controls lamellipodium formation and cell spreading. We were surprised to find that loss of α-parvin, but not that of β-parvin, markedly stimulated Rac activation and enhanced lamellipodium formation. Overexpression of β-parvin, however, was insufficient for stimulation of Rac activation or lamellipodium formation, although it was sufficient for promotion of apoptosis, another important cellular process that is regulated by PINCH-1, ILK, and α-parvin. In addition, we show that the interactions of ILK with α- and β-parvin are mutually exclusive. Overexpression of β-parvin or its CH₂ fragment, but not a CH₂ deletion mutant, inhibited the ILK-α-parvin complex formation. Finally, we provide evidence suggesting that inhibition of the ILK-α-parvin complex is sufficient, although not necessary, for promotion of apoptosis. These results identify Rac as a downstream target of PINCH-1, ILK, and parvin. Furthermore, they demonstrate that α- and β-parvins play distinct roles in mammalian cells and suggest that the formation of the ILK-α-parvin complex is crucial for protection of cells from apoptosis.

Cell-extracellular matrix (ECM) adhesion plays a pivotal role in control of cell behavior such as morphological changes and survival (1–8). At the molecular level, cell-ECM adhesion is mediated by transmembrane cell adhesion receptors (e.g. integrins) and a number of highly selective, receptor-proxi-
cells. The expression of the GST and maltose-binding protein fusion proteins was induced with isopropyl β-D-thiogalactoside, and they were purified by affinity chromatography using glutathione-Sepharose 4B and amylose-agarose, respectively.

**Generation of Monoclonal Anti-β-Parvin Antibodies**—Mouse monoclonal anti-β-parvin antibodies were prepared using a GST fusion protein containing the N-terminal region (residues 1–91) of human β-parvin as an antigen based on a method described previously (13, 24). Hybridoma supernatants were initially screened for anti-β-parvin antibody activities by enzyme-linked immunosorbent assay and Western blotting using a maltose-binding protein fusion protein containing full-length β-parvin sequence. Antibodies that recognize maltose-binding protein-β-parvin were selected and further characterized by Western blotting using extracts of cells expressing GFP- or FLAG-tagged α- and β-parvin proteins.

**Construction of Mammalian β-Parvin Expression Vectors and DNA Transfection**—To generate expression vectors encoding the N-terminal GST-tagged full-length or mutant forms of β- or α-parvin, the full-length or fragments of human β- or α-parvin cDNA were generated by PCR and cloned into the pGEM-F-C2 expression vector (BD Biosciences Clontech). To generate FLAG-β-parvin expression vectors, DNA fragments encoding the full-length (364 residues) or the short form (350 residues) (14) of β-parvin were cloned into the EcoRI/KpnI sites of the pFLAG-CMV-6C vector (Sigma). To generate expression vectors encoding untagged full-length, short or mutant forms of β-parvin, or the wild-type or mutant forms of α-parvin, DNA fragments encoding the corresponding forms of α- or β-parvin (including the stop codon) were cloned into the EcoRI/KpnI sites in the p3xFLAG-CMV-14 expression vector (Sigma). The expression vector encoding the C-terminally FLAG-tagged α-parvin was described previously (13). To express GFP- or FLAG-tagged or untagged α- or β-parvin proteins, Hela cells were transfected with the corresponding expression vectors using LipofectAMINE Plus (Invitrogen) as described previously (13, 17). The expression of the GFP- or FLAG-tagged or untagged α- or β-parvin proteins in the transfectants was confirmed by Western blotting with anti-GFP, anti-FLAG, anti-α-parvin, or anti-β-parvin antibodies as specified in each experiment.

**RNA Interference**—The sequences of β-parvin siRNAs were selected based on a method described previously (25). The 21-nucleotide synthetic siRNA duplexes were prepared by Dharmacon Research. In preliminary experiments, we screened siRNAs for their effects on suppression of β-parvin expression. We found that two siRNAs (only the sense sequences of the targeted sites are shown), 5′-AACCTGT-TCACCAAGTACAAG-3′ (β-parvin siRNA-2) and 5′-AACAAGCT- GAATTTGGAGGTG-3′ (β-parvin siRNA-3), effectively mediate the silencing of β-parvin expression. The β-parvin siRNA-3 was used in all the sequential experiments. The sequences of siRNAs that target ILK, PINCH-1, and α-parvin, respectively, were described previously (18, 26). Hela cells were transfected with siRNAs that specifically targeted β-parvin, α-parvin, PINCH-1, or ILK or were transfected with a 21-nucleotide irrelevant RNA duplex as a control, using OligoFectamine (Invitrogen). The cells were analyzed 2 days after the RNA transfection as specified in each experiment. The transfection efficiency of the siRNA experiments was at least 70% based on Western blotting analyses of the targeted proteins.

**Rac Activation Assay**—To analyze the effects of depletion of β-parvin, α-parvin, PINCH-1, or ILK on Rac activation, Hela cells were transfected with siRNAs that specifically target β-parvin, α-parvin, PINCH-1, or ILK or were transfected with a 21-nucleotide irrelevant RNA duplex as a control, using OligoFectamine (Invitrogen). Forty-eight hours after RNA transfection, the cells were harvested and then plated in 60-mm dishes pre-coated with collagen I (BD Biosciences Discovery Labware), and incubated at 37°C under a 5% CO2/95% air atmosphere for 10 min. Activated Rac was detected by using a Rac activation assay kit (Cytoselect, Inc., Denver, CO) following the manufacturer’s protocol. In brief, the cells in the 60-mm dishes were lysed with 600 μl of the lysis buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 0.3 mM NaCl, and 2% Igepal). Cell lysates were immediately centrifuged at 14,000 rpm for 5 min. The supernatants were mixed with glutathione-Sepharose beads (20 μl of beads/500 μl of cell lysate) that were immobilized with GST-fusion protein containing the p21-binding domain of PAK1 (GST-PBD) and incubated at 4°C for 45 min. At the end of incubation, the beads were washed twice with the wash buffer (25 mM Tris, pH 7.5, 30 mM MgCl2, and 40 μM NaCl), and then mixed with 30 μl of Laemmli SDS sample buffer and heated at 100°C for 5 min. Rac protein bound to the GST-PBD-containing beads (the activated Rac) was detected by Western blotting with a monoclonal anti-Rac antibody (Upstate Biotechnology). In parallel experiments, total Rac protein was detected by Western blotting analyses of the cell lysates (20 μl of cell lysates/lane) with the monoclonal anti-Rac antibody under identical conditions. The amounts of the activated and the total Rac proteins were quantified using a chemiluminescence detection system (Bio-Rad), and the ratio of the activated Rac protein versus the total Rac protein (Rac activity) was determined using the Quantity One software (Bio-Rad). The relative Rac activity in the ILK, PINCH-1, β-parvin, or α-parvin siRNA transfectants was expressed as: the Rac activity in the siRNA transfectants/the Rac activity in the control transfectants (the relative Rac activity of the control transfectants = 1).

To analyze the effect of overexpression of β-parvin on Rac activation, Hela cells were transfected with the expression vector containing the
full-length β-parvin sequence or a vector lacking β-parvin sequence as a control. The relative Rac activities in the β-parvin-overexpressing cells and the control cells were determined as described above.

**Cell Morphology and Actin Cytoskeleton Staining**—Cells (as specified in each experiment) were plated in Opti-MEM I serum-free medium (Invitrogen) on fibronectin- or collagen I-coated surface. Cell morphology was observed under an Olympus IX70 fluorescence microscope equipped with a Hoffman Modulation Contrast system and recorded with a digital camera. To visualize actin cytoskeleton, the cells were fixed with 3.7% paraformaldehyde in PBS and stained with tetramethyl rhodamine-labeled phalloidin.

**Immunoprecipitation**—Hela cells were transfected with expression vectors encoding FLAG-ILK, FLAG-PINCH-1, and/or GFP-tagged or untagged wild-type or mutant forms of α- or β-parvin, or empty vector lacking protein coding sequence as a control, as specified in each experiment. The transfectants were lysed with 1% Triton X-100 in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 2 mM Na3VO4, 100 mM NaF, and protease inhibitors. The cell lysates were mixed with agarose beads conjugated with monoclonal anti-FLAG antibody M2 (Sigma) (650 μg lysates/50 μl of 50% (v/v) M2 beads). The beads were washed four times, and the proteins bound were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. The samples were analyzed by Western blotting with antibodies as specified in each experiment.

**Apoptosis**—Hela cells were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were transfected with the expression vector encoding the wild-type or mutant forms of α- or β-parvin or a control vector lacking parvin sequences using LipofectAMINE Plus. Two days after transfection, apoptosis was analyzed using terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) and caspase-3 assays. The TUNEL assay was performed using an In Situ cell death detection kit (Roche). The caspase-3 activities were measured using fluorogenic caspase-3 substrate VII (N-acetyl-DEVD-7-amino-4-trifluoromethyl coumarin) from Calbiochem according to the manufacturer’s protocol.

**RESULTS**

**ILK and PINCH-1 Are Crucially Involved in the Rac Activation**—Activation of Rac is among the earliest cell-ECM adhesion-induced signaling events that are essential for cell spreading (22, 23). We recently found that ILK and PINCH-1 are indispensable for prompt cell spreading (18). To determine whether ILK and PINCH-1 function upstream of Rac, we transfected Hela cells with siRNAs that specifically target ILK and PINCH-1, respectively. Western blotting analyses showed that, as expected, the level of ILK (Fig. 1A, compare lanes 1 and 2), but not that of actin (Fig. 1B, compare lanes 1 and 2), was

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**Fig. 2. Characterization of the monoclonal anti-β-parvin antibody.** A, lysates (15 μg/lane) of Hela cells expressing GFP-α-parvin (lanes 1, 3, and 5) or GFP-β-parvin (lanes 2, 4, and 6) were analyzed by Western blotting with a polyclonal anti-GFP antibody (lanes 1 and 2) and monoclonal anti-β-parvin antibody 11A5 (lanes 3 and 4), and monoclonal anti-α-parvin antibody 3B5 (lanes 5 and 6), respectively. B, lysates (20 μg/lane) of Hela cells expressing a C-terminally FLAG-tagged α-parvin (lanes 1 and 3) or the control cells (lanes 1 and 3) were analyzed by Western blotting with monoclonal anti-α-parvin antibody 3B5 (lanes 1 and 3) and monoclonal anti-β-parvin antibody 11A5 (lanes 3 and 4), respectively.

**Fig. 3. α- and β-parvins are co-expressed in Hela cells.** Hela cells were transfected with a control vector lacking β-parvin sequence (lane 1) or expression vectors containing sequences of the short form of β-parvin (lane 2) or the full-length β-parvin (lane 3). The transfectants were analyzed by Western blotting with monoclonal anti-β-parvin antibody 11A5 (A) and monoclonal anti-α-parvin antibody 3B5 (B), respectively. Lane 1 was loaded with 5 μg of cell lysates; lanes 2 and 3 were loaded with 2.5 μg of cell lysates/lane.

inhibitors. The cell lysates (500 μg) were mixed with 500 μl of hybridoma culture supernatant containing monoclonal anti-α-parvin antibody 1D4. After incubation for 3 h, they were mixed with 40 μl of UltraLink Immobilized Protein G (Pierce) and then incubated for an additional 1.5 h. The beads were washed four times, and the proteins bound were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. The samples were analyzed by Western blotting with antibodies as specified in each experiment.
We next sought to test whether parvin Antibodies—depleted Hela cells with expression vectors encoding GFP-

Lucates (10 μg) of Hela cells (lane 1) and Hela cells that were transfected with the control RNA (lane 2), the β-parvin-specific siRNA (lane 3), or the α-parvin-specific siRNA (lane 4) were analyzed by Western blotting with monoclonal anti-β-parvin antibody 11A5 (A), monoclonal anti-α-parvin antibody 3B5 (B), and polyclonal anti-actin antibodies (C), respectively. D–F, overexpression of α-parvin reduces the cellular level of β-parvin. Lysates of Hela cells (lane 1) and Hela cells that were transfected with expression vectors encoding α-parvin (lane 2) or the full-length β-parvin (lane 4) or a control vector lacking parvin sequences (lane 3) were analyzed by Western blotting with monoclonal anti-α-parvin antibody 3B5 (D), anti-β-parvin antibody 11A5 (E), and polyclonal anti-actin antibodies (F), respectively. Lanes in D and F were loaded with 8 μg of proteins/lane; lanes in E were loaded with 20 μg of proteins/lane.

Generation and Characterization of Monoclonal Anti-β-Parvin Antibodies—We next sought to test whether α- and β-parvin, which are known to interact with ILK (13, 14, 30), are required for the activation of Rac. To this end, we first determined whether β-parvin is co-expressed with α-parvin in Hela cells. To facilitate this, we generated a GST fusion protein containing the N-terminal region (residues 1–91) of human β-parvin and used it to develop monoclonal anti-β-parvin antibodies as described under “Experimental Procedures.” To characterize the monoclonal anti-β-parvin antibodies, we transfected Hela cells with expression vectors encoding GFP-α-parvin and GFP-β-parvin, respectively. The expression of the N-terminally GFP-tagged α-parvin (Fig. 2A, lane 1) and β-parvin (Fig. 2A, lane 2) in the corresponding transfectants was confirmed by Western blotting with anti-GFP antibodies. GFP-β-parvin (Fig. 2A, lane 4), but not GFP-α-parvin (Fig. 2A, lane 3), was readily detected by monoclonal anti-β-parvin antibody 11A5, suggesting that it preferentially recognizes the β-parvin fusion protein. In a parallel experiment, GFP-α-parvin (Fig. 2A, lane 5), but not GFP-β-parvin (Fig. 2A, lane 6), was readily detected by monoclonal anti-α-parvin antibody 3B5 (13). To further characterize the monoclonal anti-β-parvin antibody, we transfected Hela cells with an expression vector encoding a C-terminally FLAG-tagged α-parvin and a FLAG vector lacking α-parvin sequence as a control, respectively. Overexpression of the FLAG-α-parvin protein in the FLAG-α-parvin transfecteds (Fig. 2B, lane 1), but not in the control transfecteds (Fig. 2B, lane 2), was confirmed by Western blotting with a monoclonal anti-α-parvin antibody. It is interesting that a relatively weak band that migrated to the position of FLAG-α-parvin was detected by the monoclonal anti-β-parvin antibody in the transfecteds that overexpress the C-terminally FLAG-tagged α-parvin (Fig. 2B, lane 3) but not in the control transfecteds (Fig. 2B, lane 4), suggesting that whereas the monoclonal anti-β-parvin antibody preferentially recognizes β-parvin, it can weakly recognize α-parvin protein that lacks the N-terminal GFP tag.

α- and β-Parvin are Co-expressed in Human Cells and α-Parvin Negatively Regulates β-Parvin—We next tested whether α- and β-parvin are co-expressed in human cells. To do this, we probed the lysates of Hela cells with the monoclonal anti-α-parvin and anti-β-parvin antibodies. The results showed that, as expected, the monoclonal anti-α-parvin antibody recognized a single band in Hela cells (Fig. 3B, lane 1). The monoclonal anti-β-parvin antibody, on the other hand, recognized several bands with apparent molecular masses in the range of 45–51 kDa (Fig. 3A, lane 1). To test whether any of these bands
results indicate that lane 3, which is consistent with the notion that the β-parvin(s) is produced from an active, alternative initiation codon downstream of the initiation codon nearest the 5’ end (14). A close inspection of the anti-β-parvin Western blot derived from the Hela cells (Fig. 3A, lane 1) revealed that the lowest band recognized by the monoclonal anti-β-parvin antibody migrated to the same position as β-parvin(s) (Fig. 3A, lane 2). These results indicate that β-parvin (specifically β-parvin(s)) is co-expressed with α-parvin in human Hela cells. No protein band with apparent molecular mass that is identical to that of β-parvin(l) was detected in Hela cells, suggesting that β-parvin(l) is either not expressed or is expressed at a level much lower (below the detection level under the condition used) than that of β-parvin(s), which is consistent with the finding that β-parvin(s) is preferentially expressed in cells transfected with the expression vector encoding β-parvin(l) (Fig. 3A, lane 3). As expected, neither β-parvin(s) nor β-parvin(l) that was overexpressed in the corresponding transfectants was recognized by the monoclonal anti-α-parvin antibody (Fig. 3B, lanes 2 and 3). Co-expression of α- and β-parvin proteins was also found in many other mammalian cells including human WI-38 fibroblasts, Caco-2 adenocarcinoma cells, and mouse C2C12 myoblasts (data not shown).

To confirm that the endogenous protein that co-migrated with the exogenously expressed β-parvin protein is indeed β-parvin, we transfected Hela cells with an siRNA that specifically targets β-parvin. Transfection of the cells with the β-parvin siRNA, but not that with an irrelevant small RNA, effectively depleted this protein (Fig. 4A, compare lane 3 with lanes 1 and 2). The bands that migrated slower than β-parvin(s), however, were not affected by the β-parvin siRNA (Fig. 4A, lane 3), indicating that they most probably represent proteins that share a common epitope with β-parvin but are encoded by genes other than that of β-parvin or β-parvin variants that lack the siRNA target sequence. It was striking that depletion of α-parvin (Fig. 4B, lane 4) dramatically increased the levels of both β-parvin(s) and β-parvin(l) (Fig. 4A, lane 4), indicating that the presence of α-parvin suppresses the levels of the β-parvin proteins. Depletion of β-parvin (Fig. 4A, lane 3), however, did not significantly alter the level of α-parvin (Fig. 4B, compare lane 3 with lanes 1 and 2). In control experiments, equal loading was confirmed by probing the same samples with an anti-actin antibody (Fig. 4C). To further test this, we overexpressed α-parvin in Hela cells (Fig. 4D, lane 2) and analyzed its effect on the level of β-parvin. The results showed that the level of β-parvin(s) was reduced (although it was not eliminated) in α-parvin-overexpressing cells (Fig. 4E, compare lane 2 with lanes 1 and 3). Consistent with the observation that loss of β-parvin did not significantly alter the level of α-parvin (Fig. 4B, lane 3), we did not detect significant changes of α-parvin in cells overexpressing β-parvin (Fig. 4, D and E, lane 4; also see Fig. 8C, lanes 6 and 8). Equal loading was confirmed by probing the same samples with an anti-actin antibody (Fig. 4F). Taken together, these results demonstrate that α- and β-parvin are co-expressed in human cells. Furthermore, they reveal that α-parvin negatively regulates the cellular level of β-parvin.
Loss of α-Parvin, but Not That of β-Parvin, Stimulates Rac Activation and Enhances Lamellipodium Formation—To test whether α- or β-parvin plays a role in regulation of Rac activation, we analyzed the levels of activated Rac in cells that were depleted of α- and β-parvin, respectively. The results showed that loss of α-parvin markedly stimulated Rac activation (Fig. 5). In contrast, depletion of β-parvin did not stimulate Rac activation; in fact, it was slightly reduced (Fig. 5). Consistent with the marked stimulation of Rac activation induced by the loss of α-parvin, much more extensive lamellipodia were formed in the α-parvin-deficient cells upon plating on fibronectin (Fig. 6, compare B and E with A and D) or collagen-I (data not shown). Lamellipodium formation was not significantly increased in cells that were depleted of β-parvin (Fig. 6, compare C and F with A and D), which again is consistent with the observation that depletion of β-parvin failed to stimulate Rac activation. As a whole, these results suggest that whereas loss of PINCH-1 or ILK reduces Rac activation, loss of α-parvin, but not β-parvin, increases Rac activation and lamellipodium formation.

Overexpression of β-Parvin Is Insufficient for Stimulation of Rac Activation or Lamellipodium Formation—Because depletion of α-parvin dramatically increased the levels of the short form as well as the full-length β-parvin (Fig. 4A, lane 4), we sought to test whether overexpression of β-parvin is sufficient to stimulate Rac activation and lamellipodium formation. To do this, we transfected Hela cells with the expression vector containing the full-length β-parvin sequence and a control vector lacking β-parvin sequence, respectively. Overexpression of the short form as well as the full-length β-parvin transfectants was confirmed by Western blotting with monoclonal anti-β-parvin antibody 11A5 (Fig. 7A, lane 2). The β-parvin-overexpressing cells, however, failed to exhibit an elevated level of the Rac activation (Fig. 7, D and E) or enhanced lamellipodium formation (Fig. 7, B and C). Similar results were obtained with cells transfected with the expression vector encoding the short form of β-parvin (data not shown). Taken together, these results suggest that overexpression of β-parvin, unlike depletion of α-parvin, is insufficient for stimulation of Rac activation and lamellipodium formation.

β-Parvin Negatively Regulates the Complex Formation between α-Parvin and ILK—Because α- and β-parvin are structurally closely related to each other and both proteins can bind to ILK, we tested whether overexpression of β-parvin affects the binding of α-parvin to ILK. To do this, we expressed FLAG-tagged ILK (Fig. 8A, lanes 6 and 7), which facilitated immunoprecipitation of ILK, in cells overexpressing β-parvin (Fig. 8B, lane 6) as well as in cells expressing a normal level of β-parvin (Fig. 8B, lane 7). FLAG-ILK was immunoprecipitated with monoclonal anti-FLAG antibody M2. Western blotting analyses of the immunoprecipitates showed that similar amounts of FLAG-ILK were precipitated from the β-parvin-overexpressing cells and the control cells (Fig. 8A, compare lanes 1 and 2). Consistent with this, similar amounts of PINCH-1 were detected in the FLAG-ILK immunoprecipitates derived from the β-parvin-overexpressing cells and the control cells (Fig. 8D, lanes 1 and 2). However, the amount of α-parvin bound to FLAG-ILK was noticeably reduced in the β-parvin-overexpressing cells and the control cells (Fig. 8D, lanes 1 and 2). Consistent with this, similar amounts of PINCH-1 were detected in the FLAG-ILK immunoprecipitates derived from the β-parvin-overexpressing cells and the control cells (Fig. 8D, lanes 1 and 2). However, the amount of α-parvin bound to FLAG-ILK was noticeably reduced in the β-parvin-overexpressing cells (Fig. 8C, compare lane 1 with lane 2), whereas in the same cells, more β-parvin was found to complex with FLAG-ILK (Fig. 8B, compare lane 1 with lane 2). These results suggest that overexpression of β-parvin increases the amount of α-parvin bound to FLAG-ILK and concomitantly reduced the amount of α-parvin bound to endogenous ILK, we expressed FLAG-tagged PINCH-1 (Fig. 8D, lanes 8 and 9) in cells that express different amounts of...
Distinct Roles of α- and β-Parvins

Fig. 8. Overexpression of β-parvin inhibits the ILK-α-parvin complex formation. Lysates (20 μg/lane in A, B, and D and 10 μg/lane in C) of Hela cells transfected with the FLAG-ILK expression vector and the β-parvin (I) expression vector (lane 6), the FLAG-ILK expression vector and a control vector lacking β-parvin sequence (lane 7), the FLAG-PINCH-1 expression vector and the β-parvin (I) expression vector (lane 8), the FLAG-PINCH-1 expression vector and a control vector lacking β-parvin sequence (lane 9), or the control vector alone (lane 10) were analyzed by Western blotting with mouse monoclonal anti-ILK antibody 65.1 (A), mouse monoclonal anti-β-parvin antibody 11A5 (B), mouse monoclonal anti-α-parvin antibody 3B5 (C), and a rabbit polyclonal anti-PINCH-1 antibody (D), respectively. The cell lysates were mixed with agarose beads conjugated with a monoclonal anti-FLAG antibody (M2). After washing, the anti-FLAG immunoprecipitates (lanes 1-5 as specified in the figure) were analyzed by Western blotting with mouse monoclonal anti-ILK antibody 65.1 (A), mouse monoclonal anti-β-parvin antibody 11A5 (B), mouse monoclonal anti-α-parvin antibody 3B5 (C), and a rabbit polyclonal anti-PINCH-1 antibody (D), respectively. The asterisk (lane 5) indicates the position of mouse IgG heavy chain released from the M2 beads that was recognized by the HRP-conjugated anti-mouse IgG (A-C) but not anti-rabbit IgG (D) secondary antibodies.

β-parvin (Fig. 8B, lanes 8 and 9). A similar amount of endogenous ILK (Fig. 8A, lanes 3 and 4) was co-immunoprecipitated with FLAG-PINCH-1 (Fig. 8D, lanes 3 and 4) in cells overexpressing β-parvin as well as in cells expressing a normal level of β-parvin. The amount of β-parvin bound to the endogenous ILK protein was significantly increased in cells overexpressing β-parvin (Fig. 8B, compare lane 3 with lane 4). The amount of α-parvin bound to the endogenous ILK protein, on the other hand, was noticeably reduced in cells overexpressing β-parvin (Fig. 8C, compare lane 3 with lane 4). Taken together, these results suggest that overexpression of β-parvin reduces the complex formation between ILK and α-parvin.

How does overexpression of β-parvin inhibit the complex formation between ILK and α-parvin? Because both α- and β-parvin can bind to ILK (13, 14, 30) and the two parvin proteins (including the C-terminal CH2 domains that mediate the ILK binding) share a high degree of structural similarity, one possible mechanism is that ILK does not bind to α- and β-parvin simultaneously; therefore, overexpression of β-parvin, which increases the amount of β-parvin bound to ILK (Fig. 8B), reduces the amount of α-parvin bound to ILK. To test experimentally whether ILK binds to α- and β-parvin simultaneously, we immunoprecipitated α-parvin (Fig. 9A, lane 2) and ILK that bound to α-parvin (Fig. 9A, lane 6) from Hela cells. Western blotting analyses of the anti-α-parvin immunoprecipitates showed that β-parvin(s), which was the only form of β-parvin that was detectable in Hela cells (Fig. 4), was not associated with the α-parvin-ILK complex (Fig. 9A, lane 4), supporting the notion that β-parvin(s) and α-parvin do not bind to ILK simultaneously. However, a band with apparent molecular mass identical to that of α-parvin was detected in the anti-α-parvin immunoprecipitates by the monoclonal anti-β-parvin antibody (Fig. 9A, lane 4). Because 1) the monoclonal anti-β-parvin antibody possesses low cross-activity toward N-
terminally untagged α-parvin (Fig. 2B, lane 3), 2) α-parvin was highly enriched in the anti-α-parvin immunoprecipitates (Fig. 9A, lane 2), and 3) the apparent molecular mass of the band recognized by the monoclonal anti-β-parvin antibody is identical to that of α-parvin (Fig. 9A, compare lanes 2 and 4), this band most likely represents the immunoprecipitated α-parvin protein that was cross-recognized by the monoclonal anti-β-parvin antibody. Alternatively (although much less likely given that β-parvin(1) was undetectable in Hela cell lysates), this band could represent β-parvin(1) (which is only slightly smaller than α-parvin) associated with the α-parvin-ILK complex. To test whether β-parvin(1) could interact with ILK that was bound to α-parvin, we expressed a FLAG-tagged β-parvin(1), which allowed us to detect it using a monoclonal anti-FLAG antibody that does not at all recognize α-parvin, in Hela cells. α-Parvin (Fig. 9B, lane 2), together with ILK (Fig. 9B, lane 6) that was associated with α-parvin, was immunoprecipitated from the cells with the monoclonal anti-α-parvin antibody. Western blotting analysis with the monoclonal anti-FLAG antibody showed that, despite the presence of abundant FLAG-β-parvin(1) in the cell lysates (Fig. 9B, lane 3), the long form of β-parvin (Fig. 9B, lane 4) was unable to bind to ILK (Fig. 9B, lane 6) that was associated with α-parvin (Fig. 9B, lane 2). Taken together, these results suggest that neither β-parvin(s) nor β-parvin(1) binds simultaneously with α-parvin to ILK.

To further test the hypothesis that β-parvin inhibits the complex formation between ILK and α-parvin by competing with α-parvin for ILK binding, we analyzed the effect of overexpression of the ILK binding CH2 domain of β-parvin on the formation of the ILK-α-parvin complex. If β-parvin indeed functions in the regulation of the ILK-α-parvin complex formation through its binding to ILK, overexpression of the ILK-binding CH2 fragment should, like that of the wild-type β-parvin, inhibit the ILK-α-parvin complex formation. To facilitate the detection of the CH2 fragment (our monoclonal anti-β-parvin antibody recognizes the N-terminal region but not the C-terminal CH2 domain of β-parvin), we fused the CH2 domain to the C terminus of GFP. Hela cells were co-transfected with expression vectors encoding FLAG-PINCH-1 and GFP-CH2 or GFP as a control. The expression of GFP-CH2 (Fig. 10A, lane 1) and GFP (Fig. 10A, lane 2) in the corresponding transfectants was confirmed by Western blotting with an anti-GFP antibody. As expected, ILK (Fig. 10C, lanes 3 and 4) was readily co-immunoprecipitated with FLAG-PINCH-1 (Fig. 10C, lanes 3 and 4) from both the GFP-CH2-expressing and the control cells. Probing the same samples showed that GFP-CH2 (Fig. 10A, lane 3), but not GFP (Fig. 10A, lane 4), was co-immunoprecipitated with ILK and FLAG-PINCH-1. By contrast, the amount of α-parvin that was co-immunoprecipitated was significantly reduced in the GFP-CH2-expressing cells (Fig. 10D, compare lanes 3 and 4). Thus, as predicted by the direct competition mechanism, overexpression of the ILK binding CH2 domain of β-parvin, like that of the wild-type β-parvin (Fig. 8), inhibits the complex formation between ILK and α-parvin. Consistent with this model, overexpression of the β-parvin CH2 fragment also inhibited the binding of the endogenous β-parvin to ILK (Fig. 10E, compare lanes 3 and 4).

Another prediction of our model is that overexpression of mutant forms of β-parvin that do not bind to ILK, unlike that of the wild-type or the CH2 domain of β-parvin, should not inhibit the ILK-α-parvin complex formation. To test this, we overexpressed an ILK-binding defective β-parvin mutant in which part of the CH2 domain was deleted (∆292), and the wild-type β-parvin as a positive control, in Hela cells. The complex formation between ILK and α-parvin was analyzed as described above. As expected, ILK (Fig. 11B, lane 5) and α-parvin.
Distinct Roles of α- and β-Parvins

Inhibition of the Complex Formation between ILK and α-Parvin Promotes Apoptosis—We next sought to test whether overexpression of β-parvin (and consequently reducing the ILK-α-parvin complex formation) alters cell survival, another important cellular process that is regulated by α-parvin and ILK. To do this, we overexpressed β-parvin, and α-parvin as a control, in Hela cells. Analyses of the activity of caspase-3, a key mediator of apoptosis, showed that overexpression of β-parvin, but not that of α-parvin, significantly increased caspase-3 activity (Fig. 12A). To confirm the pro-apoptotic effect, we analyzed the cells by TUNEL. The results showed that, consistent with the increase in caspase-3 activity, overexpression of β-parvin (Fig. 12, F, G, and J), but not that of α-parvin (Fig. 12, D, E, and J), significantly increased the percentage of TUNEL-positive (apoptotic) cells. It is interesting that overexpression of Δ292, which does not inhibit the ILK-α-parvin complex formation, also increased caspase-3 activity (Fig. 12A) and the percentage of TUNEL positive cells (Fig. 12, H–J).

To further test whether inhibition of the ILK-α-parvin complex formation is sufficient for promoting apoptosis, we analyzed the effect of overexpression of the CH2 domain of β-parvin on apoptosis. We reasoned that if the ILK-α-parvin complex is indeed crucial for protection of cells from apoptosis, overexpression of the β-parvin CH2 domain, which effectively inhibits the ILK-α-parvin complex formation (Fig. 10), should also result in an increase of apoptosis. To do this, we overexpressed GFP-CH2, and GFP alone as a control, in Hela cells and analyzed apoptosis using both the caspase-3 and TUNEL assays. The results showed that overexpression of GFP-CH2, but not that of GFP, significantly increased the caspase-3 activity (Fig. 12K). Consistent with this, overexpression of GFP-CH2, but not that of GFP, also significantly increased the percentage of TUNEL-positive cells (Fig. 12, L–P), confirming that the formation of the ILK-α-parvin complex is crucial for protection of cells from apoptosis.

Fig. 11. The ILK-binding CH2 domain is required for β-parvin-induced inhibition of the ILK-α-parvin complex formation. Hela cells were transfected with expression vectors encoding FLAG-PINCH-1, the ILK-binding defective β-parvin mutant in which residues 292–364 were deleted (Δ292), or full-length β-parvin as indicated in the figure. The cell lysates were mixed with agarose beads conjugated with monoclonal anti-FLAG antibody M2. The cell lysates (lanes 1–4; 15 μg/lane in A and D and 20 μg/lane in B and C) and anti-FLAG immunoprecipitates (lanes 5–8) were analyzed by Western blotting with a rabbit polyclonal anti-FLAG antibody (A), mouse monoclonal anti-ILK antibody 661.1 (B), mouse monoclonal anti-β-parvin antibody 11A5 (C), and mouse monoclonal anti-α-parvin antibody 3B5 (D), respectively. In B, the band that migrated slightly more slowly than ILK, which was labeled with an asterisk in lane 8, represents the mouse IgG heavy chain derived from the mouse monoclonal anti-FLAG antibody in the immunoprecipitates.

Distinct Roles of α- and β-Parvins

The work presented in this article provides important new information on the assembly and functions of the PINCH-ILK-parvin complexes. By using monoclonal anti-α-parvin and anti-β-parvin antibodies, we have demonstrated that the two structurally closely related parvin proteins are co-expressed in human cells. Furthermore, we have shown that β-parvin, like α-parvin, forms a ternary complex with ILK and PINCH-1 in cells. It is noteworthy that although both α- and β-parvin can bind to ILK, they do not bind to ILK simultaneously. Thus, in cells that express both α- and β-parvin (e.g. Hela cells), at least two types of ILK-parvin complexes exist, one containing α-parvin and the other containing β-parvin. The relative levels (or the ratio) of the ILK-α-parvin complex and the ILK-β-parvin complex are controlled, at least in part, by the expression levels of α- and β-parvin, as an elevation of the expression level of β-parvin effectively increases the level of the ILK-β-parvin complex and concomitantly decreases the level of the ILK-α-parvin complex. β-Parvin, in turn, can be regulated by α-parvin, as depletion of α-parvin significantly increases the level of β-parvin, whereas overexpression of α-parvin reduces the level of β-parvin. The mutual regulation between α- and β-parvins provides a feedback mechanism by which cells can precisely control the relative levels of these two structurally closely related proteins and consequently the levels of protein complexes containing α- or β-parvin.

Why do cells need to control the relative levels of α- or β-parvin? The studies presented in this article demonstrate that, despite possessing identical domain architecture and sharing a high level of sequence similarity, α- and β-parvin play distinct and sometime even contrasting roles in regulation...
of cell behavior. For example, we have found that overexpression of β-parvin, but not that of α-parvin, promotes apoptosis (Fig. 12). Consistent with the pro-apoptotic role of β-parvin, we have found that depletion of β-parvin in Hela cells, unlike that of α-parvin (26), did not increase apoptosis. Thus, in contrast to α-parvin that functions as an important anti-apoptotic protein (26), β-parvin functions as a pro-apoptotic protein. Given the facts that all three components of the PINCH-ILK-α-parvin complex are anti-apoptotic (18, 26, 31, 32) and overexpression of β-parvin inhibits the complex formation between ILK and α-parvin (Fig. 8), the pro-apoptotic effect induced by the overexpression of β-parvin most likely results from, at least in part, its inhibition of the ILK-α-parvin complex. In fact, overexpression of the ILK-binding CH2 domain of β-parvin alone is sufficient for promoting apoptosis (Fig. 12, K–P), providing additional evidence supporting a crucial role of the ILK-α-parvin complex in protection of cells from apoptosis. Although inhibition of the ILK-α-parvin complex formation is sufficient for promoting apoptosis, it is not necessary for promoting apoptosis, because overexpression of Δ292, which does not inhibit the ILK-α-parvin complex formation (Fig. 11), also significantly increased apoptosis (Fig. 12). Because Δ292 shares significant sequence similarity with the N-terminal region and the CH1 domain of α-parvin, it is attractive to propose that Δ292 promotes apoptosis by preventing binding of other yet to be identified proteins to the CH1 and/or the N-terminal region of α- or β-parvin. Identification of proteins that interact with the CH1 and/or the N-terminal region of α- or β-parvin will be an important and likely fruitful area of future studies.

In addition to playing contrasting roles in apoptosis, the functions of α- and β-parvins in cell shape modulation are also clearly distinct. We previously showed that both PINCH-1 and ILK are indispensable for prompt cell spreading (18). The results obtained from this study demonstrate that both PINCH-1 and ILK are required for optimal activation of Rac. Because Rac activation is essential for lamellipodium formation and cell spreading (for reviews, see Refs. 21–23), PINCH-1 and ILK probably function in the control of cell morphology by regulating, among other things, the activation of Rac. This places PINCH-1 and ILK upstream of Rac in the signaling pathways that control cell spreading. It has been well established that Rac activation is positively regulated by specific guanine nucleotide exchange factors and negatively regulated by specific GTPase-activating proteins (21–23). One of the Rac guanine

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*Y. Zhang, K. Chen, and C. Wu, unpublished observations.*
Distinct Roles of α- and β-Parvins

nucleotide exchange factors, Pak-interacting exchange factor α (or α-PIX; also known as ARHGEF6 or Cool-2), can directly bind to β-parvin (33). The binding of β-parvin to α-PIX provides a potential mechanism by which PINCH-1 and ILK could regulate Rac activation. It is worth noting, however, that although depletion of PINCH-1 or ILK reduced Rac activation by ~50–55% (Fig. 1F), depletion of β-parvin only slightly reduced Rac activation (Fig. 5C). These results suggest that PINCH-1 and ILK probably regulate Rac activation through not only β-parvin but also other proteins. It is interesting to note in this regard that PINCH-1 interacts with Nck-2 (34, 35), which can in turn bind to DOCK180 (36), another important guanine

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Distinct Roles of Two Structurally Closely Related Focal Adhesion Proteins, α-Parvins and β-Parvins, in Regulation of Cell Morphology and Survival
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