Molecular Dissection of PINCH-1 Reveals a Mechanism of Coupling and Uncoupling of Cell Shape Modulation and Survival*

Received for publication, April 18, 2005, and in revised form, May 31, 2005
Published, JBC Papers in Press, June 7, 2005, DOI 10.1074/jbc.M504189200

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How cells couple and uncouple regulation of cellular processes such as shape change and survival is an important question in molecular cell biology. PINCH-1, a widely expressed protein consisting of five LIM domains and a C-terminal tail, is an essential focal adhesion protein with multiple functions including regulation of the integrin-linked kinase (ILK) level, cell shape, and survival signaling. We show here that the LIM1-mediated interaction with ILK regulates all these three processes. By contrast, the LIM4-mediated interaction with Nck-2, which regulates cell morphology and migration, is not required for the control of the ILK level and survival. Remarkably, a short 15-residue tail C-terminal to LIM5 is required for both cell shape modulation and survival, albeit it is not required for the control of the ILK level. The C-terminal tail not only regulates PINCH-1 localization to focal adhesions but also functions after it localizes there. These findings suggest that PINCH-1 functions as a molecular platform for coupling and uncoupling diverse cellular processes via overlapping but yet distinct domain interactions.

Cell-extracellular matrix (ECM) adhesion is a fundamental process that is critically involved in embryonic development and numerous physiological and pathological processes including injury repair, cancer, and organ (e.g. heart and renal) failure. Among key regulators of cell-ECM adhesion and signaling are proteins localized at the membrane-actin cytoskeleton junctions in cell-ECM contacts (e.g. focal adhesions). PINCH-1 is a widely expressed focal adhesion protein consisting of five LIM domains and a short residue C-terminal tail (for review, see Refs. 1–4). Recent biochemical, cell biological, and genetic studies have demonstrated that PINCH-1 is crucial for cell shape modulation and signal transduction (5–13). Furthermore, there is evidence suggesting that PINCH-1 could serve as a useful target for therapeutic intervention of human diseases such as cancer and organ (e.g. heart and renal) failure (2, 4, 14–19). Elucidating the molecular mechanism whereby PINCH-1 functions, therefore, is important for understanding the general principle that governs the regulation of cell-ECM adhesion and signaling as well as for the development of new therapeutic approaches that control these processes.

PINCH-1 interacts with ILK (5, 20, 21), an equally widely expressed focal adhesion component that is essential for integrin-mediated cell-ECM adhesion and signaling (for review, see Refs. 2–4, 22, and 23). The ILK-binding site has been mapped to the second zinc finger of the N-terminal-most LIM1 domain that contains Gln-40 (20, 21, 24). In addition, PINCH-1 interacts with Nck-2 (25, 26), an SH2/SH3-containing adaptor protein that is involved in both cell adhesion- and growth factor-mediated signaling and actin cytoskeleton remodeling (27–29). The Nck-2-binding site is located in the first zinc finger of the PINCH-1 C-terminal LIM4 domain (25, 26). Recently, we have suppressed the expression of PINCH-1 and ILK, respectively, in mammalian cells using RNA interference. Cells in which the expression of PINCH-1 or ILK is suppressed exhibit similar defects in cell spreading and survival (9, 30). Remarkably, depletion of PINCH-1 from mammalian cells resulted in marked down-regulation of the protein level but not the mRNA level of ILK (9). These results suggest that PINCH-1 functions at least in part by controlling the cellular level of ILK. Meanwhile, they have raised several new questions. First, does PINCH-1 function in the control of the ILK protein level via its interaction with ILK? Although the current model favors a positive answer to this question, it is important to unequivocally test this via experimentation. Second, because cell shape can influence cell survival and vice versa, is the cell survival defect induced by the depletion of PINCH-1 a simple reflection of its inhibition of cell spreading and vice versa? In other words, can the functions of PINCH-1 in cell spreading and survival be uncoupled (or separately regulated)? Third, are there PINCH sites besides the ILK binding LIM1 domain that are essential for PINCH-1-mediated cell shape modulation and survival? If there are, where are they located and do they regulate cell shape and survival via control of the level or localization of ILK? To address these questions we have used a structure-based genetic rescue approach to dissect the functions of PINCH-1. The results not only provide important information on the molecular basis of PINCH-1-mediated processes but also shed new light on the mechanism that controls the coupling and uncoupling of diverse processes such as shape modulation and survival.

Experimental Procedures

Antibodies and Other Reagents—Rabbit antibodies against AKT, phosho-Akt (Ser-473), and phosho-Akt(Thr-308) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal anti-paxillin antibody (clone 349) was from BD Transduction Labora-
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RESULTS

PINCH-1 Functions in the Maintenance of the ILK Protein Level via Its Interaction with ILK—Consistent with the previous studies (9), suppression of PINCH-1 expression in HeLa cells (Fig. 1A, compare lane 2 with lane 1) significantly reduced the protein level of ILK (Fig. 1C, compare lane 2 with lane 1). To test whether PINCH-1 functions in the maintenance of the ILK protein level via binding to ILK, we expressed the wild type PINCH-1 (Fig. 1A, lane 4) and an ILK binding-defective PINCH-1 mutant in which Gln-40 located at the ILK binding interface was replaced with alanine (Fig. 1A, lane 3) in PINCH-1 knockdown cells. The Q40A mutation ablates the ILK binding but does not alter the overall structure of LIM1 (21,
Western blotting analyses showed that expression of the wild type PINCH-1 but not that of the ILK binding-defective point mutant restored the level of ILK (Fig. 1C, lanes 3 and 4). In parallel experiments, an equal amount of actin was detected in all cell lysates (Fig. 1B). These results confirm the specificity of the ILK down-regulation induced by the PINCH-1 siRNA. Furthermore, they demonstrate that PINCH-1 functions in the maintenance of the ILK level through its interaction with ILK.

Expression of the Wild Type PINCH-1, but Not the ILK Binding-defective Point Mutant, in PINCH-1 Knockdown Cells Rescues the Defects in Cell Spreading and Survival—We next sought to identify the PINCH sites that are involved in the regulation of cell spreading and survival signaling. To this end, we first compared the effects of the wild type PINCH-1 and the ILK binding-defective point mutant on cell spreading. Unlike cells expressing a normal level of PINCH-1 (Fig. 1, D and H), PINCH-1 knockdown cells exhibited impaired ability to spread (Fig. 1, E and H). Re-expression of the wild type PINCH-1 (Fig. 1, G and H) but not that of the ILK binding-defective point mutant (Fig. 1, F and H) significantly improved the ability of cells to spread in response to cell-ECM adhesion. These results indicate that the interaction with ILK is essential for PINCH-1-mediated cell spreading.

To assess the significance of the ILK binding in cell survival signaling, we analyzed the activating phosphorylation of Akt, a key signaling intermediate that protects cells from apoptosis (35–37). Consistent with previous studies (9), knockdown of PINCH-1 (Fig. 2D, lanes 9 and 10) markedly inhibited the insulin-like growth factor 1-induced phosphorylation of Akt at Ser-473 (Fig. 2A, compare lane 10 with lane 2) and Thr-308 (Fig. 2B, compare lane 10 with lane 2). Knockdown of PINCH-1 also inhibited the Ser-473 and Thr-308 phosphorylation of Akt in cells culturing in normal serum-containing medium (see Fig. 4). Transient expression of the wild type PINCH-1 (Fig. 2D, lanes 7 and 8), but not that of the ILK binding-defective Q40A point mutant (Fig. 2D, lanes 3 and 4), restored to a considerable extent the phosphorylation of Akt (Figs. 2, A and B, lanes 4 and 8), underscoring the importance of the ILK binding in the activating phosphorylation of Akt.

To further analyze this, we measured the activity of caspase-3, a key mediator of apoptosis, in PINCH-1 knockdown cells as well as in cells that re-express the wild type or the ILK binding-defective point mutant of PINCH-1. As expected, the level of caspase-3 activity was elevated in PINCH-1 knockdown cells (Fig. 3A, Vector/PINCH-1(−)). Transient expression of the wild type PINCH-1 (Fig. 3A, WT/PINCH-1(−)) but not that of the ILK binding-defective Q40A point mutant (Fig. 3A, Q40A/PINCH-1(−)) significantly reduced the caspase-3 activity. Consistent with this, the percentage of apoptotic cells measured by TUNEL was significantly increased in the PINCH-1 knockdown cells (Fig. 3B). Transient expression of the wild type PINCH-1 but not that of the ILK binding-defective Q40A point mutant in the PINCH-1 knockdown cells significantly reduced the percentage of apoptotic cells (Fig. 3B). These results suggest that the interaction of PINCH-1 with ILK is crucial for protection of cells from apoptosis.

The Nck-2 Binding Is Dispensable for PINCH-1-mediated Cell Survival Signaling—In addition to interacting with ILK through the N-terminal-most LIM1 domain, PINCH-1 interacts with Nck-2, an SH2/SH3-containing adaptor protein, via its C-terminal LIM4 domain (25, 26). It is increasingly clear that the interaction between PINCH-1 and Nck-2 is involved in regulation of actin cytoskeleton remodeling (25, 26, 28, 38, 39). It has not been determined, however, whether or not the binding of PINCH-1 to Nck-2 plays a role in PINCH-1-mediated cell survival signaling. To test this we expressed a Nck-2 binding-defective PINCH-1 mutant (RRAA) in which Arg-197—Arg-198, located at the Nck-2 binding interface (26), were replaced with alanine residues in PINCH-1 knockdown cells (Fig. 2D, lanes 5 and 6). The R197A/R198A mutations ablate the Nck-2 binding but do not alter the overall structure of the LIM4 domain (26, 39). Transient expression of the Nck-2 binding-defective mutant (Figs. 2, A and B, lane 6), like that of the wild type PINCH-1 (Figs. 2, A and B, lane 8), significantly enhanced phosphorylation of Akt. Furthermore, it substantially reduced the activity of caspase-3 that was induced by the depletion of PINCH-1 (to a level that was comparable with what was seen in cells transiently expressing the wild type PINCH-1) (Fig. 3A, RRAA/PINCH-1(−)). TUNEL analyses confirmed that the Nck-2 binding-defective mutant effectively rescued the survival defect induced by the depletion of PINCH-1 (Fig. 3B). These results suggest that the interaction of PINCH-1 with Nck-2, unlike that with ILK, is dispensable for cell survival signaling.

The PINCH-1 C-terminal Region Is Crucial for Survival Signaling and Cell Spreading but Not Maintenance of the ILK Level—We next expressed a PINCH-1 mutant (ΔLIM5) in...
which the C-terminal LIM5 and non-LIM tail were deleted in PINCH-1 knockdown cells. The wild type PINCH-1 was expressed in PINCH-1 knockdown cells as a positive control. The expression of ΔLIM5 (Fig. 4A, lane 4) and the wide type PINCH-1 (Fig. 4A, lane 3) in the PINCH-1 knockdown cells (Fig. 4A, lane 2) was confirmed by Western blotting with an anti-PINCH-1 antibody. Probing the cells with antibodies specific for phospho-Akt (Ser-473) or phospho-Akt (Thr-308) showed that the level of Ser-473- and Thr-308-phospho-Akt, as expected, was significantly reduced in PINCH-1 knockdown cells (Figs. 4, D and E, compare lane 2 with lane 1). Whereas expression of the wild type PINCH-1 restored the level of phospho-Akt near what was found in the control cells (Figs. 4, D and E, compare lane 3 with lane 1), expression of ΔLIM5 failed to rescue the defect in Akt phosphorylation (Figs. 4, D and E, compare lane 4 with lane 2). In control experiments neither the total Akt protein level (Fig. 4F, lanes 1–4) nor the actin level (Fig. 4B, lanes 1–4) was altered, confirming the specificity of the rescue experiments. Consistent with the defect in Akt phosphorylation, expression of ΔLIM5 failed to inhibit the activation of caspase-3 induced by the depletion of PINCH-1 (Fig. 3C). These results suggest that the C-terminal region of PINCH-1, like the N-terminal LIM1, is required for cell survival signaling. Importantly, although ΔLIM5 failed to rescue the defect in survival signaling, expression of ΔLIM5, like that of the wild type PINCH-1, restored the level of ILK protein (Fig. 4C, lanes 3 and 4).

To further analyze this we expressed a PINCH-1 mutant containing all five LIM domains but lacking the C-terminal-most non-LIM tail in PINCH-1-deficient cells (Fig. 4A, lane 5). The expression of the C-terminal-most tail deletion mutant (CD) restored the level of ILK protein (Fig. 4C, lane 5) but not that of Akt phosphorylation (Figs. 4, D and E, lanes 5). Consistent with this, expression of the CD mutant failed to significantly reduce the activation of caspase-3 (Fig. 3C) and the percentage of the TUNEL-positive (apoptotic) cells (Fig. 3D) induced by the depletion of PINCH-1.

We next assessed the role of the C-terminal region in cell spreading. The results showed that expression of ΔLIM5 (Fig. 5, D and G) or CD (Fig. 5, E and G), unlike that of the wild type PINCH-1 (Fig. 5, C and G), failed to rescue the cell-spreading defect induced by the depletion of PINCH-1 (Fig. 5, B and G). Thus, despite their ability to restore ILK protein level, neither ΔLIM5 nor CD was able to rescue the defect in cell shape modulation induced by the depletion of PINCH-1. This result together with those described earlier suggests that PINCH-1 not only functions in the control of the ILK level (which is mediated by its interaction with ILK) but also is directly involved in the cell shape modulation and survival signaling (which requires both the N-terminal LIM1 domain and the C-terminal region).

The C-terminal-most Tail Not Only Regulates PINCH-1 Localization to Cell-ECM Adhesions but Also Is Required for PINCH-1 Functions at Cell-ECM Adhesions—Proper subcellular localization is critical for normal protein functions. Thus, the failure of the PINCH-1 C-terminal-most tail deletion mu-
tant to rescue cell survival and spreading defects could result from its deficiency in focal adhesion localization. To compare the ability to localize to cell-ECM adhesions, we expressed GFP-tagged wild type PINCH-1 (Fig. 6B, lane 2) and the CD mutant (Fig. 6B, lane 3) in HeLa cells and plated them on fibronectin-coated surface. Immunofluorescent staining of the cells with a monoclonal anti-ILK antibody showed that, as expected, GFP-PINCH-1 was readily co-clustered with ILK in cell-ECM adhesions (Fig. 6C and D). By contrast, the ability of the C-terminal tail deletion mutant to localize to cell-ECM adhesions was compromised (Fig. 6E and F). The reduced efficiency of the C-terminal tail deletion mutant to localize to cell-ECM adhesions was not caused by a general deficiency of cell-ECM adhesions, as abundant cell-ECM adhesions were detected in these cells (Fig. 6F). Similar results were obtained by immunofluorescent staining of the cells with a monoclonal antibody to paxillin, another marker of cell-ECM adhesions (data not shown). These results suggest that the C-terminal non-LIM tail plays an important role in the regulation of PINCH-1 localization to cell-ECM adhesions.

To test whether the C-terminal non-LIM tail is merely involved in the regulation of cell-ECM adhesion localization or it is also required for the functions of PINCH-1 after its arrival at cell-ECM adhesions, we replaced the PINCH-1 C-terminal non-LIM tail with that of PINCH-2 (40, 41) (Fig. 6A). HeLa cells were transfected with the expression vector encoding the GFP-tagged C-terminal tail swap mutant (CS). The expression of GFP-CS was confirmed by Western blotting (Fig. 6B, lane 4). Immunofluorescent analyses showed that GFP-CS (Fig. 6G), like the wild type PINCH-1, readily localized to cell-ECM adhesions where abundant ILK (Fig. 6H) and paxillin (not shown) were detected.

To test whether the C-terminal tail-swap mutant could functionally substitute PINCH-1 in cell survival signaling and shape modulation, we expressed it in PINCH-1 knockdown cells (Fig. 4A, lane 6). Expression of the CS mutant, as expected, effectively relieved the suppression of the ILK level induced by the depletion of PINCH-1 (Fig. 4C, compare lane 6 with lane 2). However, despite its ability to restore the ILK level and to localize to cell-ECM adhesions, the CS mutant failed to rescue the defect in Akt phosphorylation induced by the depletion of PINCH-1 (Figs. 4, D and E, lane 6). Consistent with this, the caspase-3 activity level remained elevated (Fig. 3C), and the percentage of apoptotic (TUNEL positive)
cells was not significantly reduced (Fig. 3D) after the expression of the CS mutant in the PINCH-1 knockdown HeLa cells. Comparison of cell spreading showed that the CS mutant, like the CD mutant, failed to rescue the cell spreading defect induced by the depletion of PINCH-1 in HeLa cells (Fig. 5, F and G). These results suggest that the PINCH-1 C-terminal-most tail functions not only in the efficient localization of PINCH-1 to cell-ECM adhesions but also in cell shape modulation and survival signaling after PINCH-1 localizes to the adhesion sites.

**DISCUSSION**

One of the key questions in molecular cell biology is how cells couple and uncouple diverse cellular processes such as shape modulation and survival. PINCH-1 is a widely expressed focal adhesion adaptor that is required for multiple processes including regulation of the ILK level, modulation of cell shape, and transduction of survival signals (9, 13). Using a structure-based reverse genetic rescue approach, we have molecularly dissected the functions of PINCH-1. The results reveal a mechanism in which PINCH-1, through its interactions with distinct binding partners, serves as a molecular platform for coupling and uncoupling diverse cellular processes via distinct domain-domain interactions.

By expression of an ILK binding-defective PINCH-1 point mutant (Q40A) in PINCH-1 knockdown cells, we have demonstrated that PINCH-1 function in the maintenance of the ILK protein level through its interaction with ILK. This result together with our previous finding that down-regulation of the ILK level is mediated at least in part by proteasome-mediated protein degradation (9) suggests that the binding of PINCH to the ankyrin domain of ILK probably helps to stabilize ILK and, hence, prevents its degradation. This may provide an explanation as to why the formation of the PINCH-1-ILK complex occurs early and precedes their localization to cell-ECM adhesions (24). Elevation of ILK levels is closely associated with and probably is an important causal factor for the progression of several human diseases including cancers and renal failure (for review, see Refs. 2, 4, 16, 19, and 42). The importance of the PINCH-1 binding to the maintenance of the ILK level suggests that it could serve as a useful target for the therapeutic control of the ILK protein level and, hence, the progression of human diseases involving abnormal expression of ILK.

A second important finding of the current study is that the cell spreading defect induced by the loss of PINCH-1 can be uncoupled from the survival defect induced by the loss of PINCH-1 and vice versa. PINCH-1 is required for both cell shape modulation and survival signaling (9, 13). We previously found that the interaction of PINCH-1 with Nck-2 functions in actin cytoskeletal regulation (39). The data presented in this paper demonstrate that the interaction with Nck-2 is not required for cell survival (Figs. 2 and 3). Thus, although PINCH-1 is intimately involved in the regulation of both the actin cytoskeleton and survival signaling, they can be uncoupled from each other. Based on these results, it is attractive to propose a model in which cells can either coordinate or separately regulate the morphological change and survival. Down-regulation of the interaction of PINCH-1 with ILK inhibits cell spreading as well as survival signaling, resulting in coordinated regulation of these two processes. On the other hand, down-regulation of the interaction of PINCH-1 with Nck-2 inhibits cell spreading and migration but not survival and, hence, the uncoupling of these two processes. The ability of PINCH-1 to either coordinate or separately regulate morphological change and survival through interactions mediated by distinct sites provides a versatile system for cells to control these processes.

The studies presented in this paper have identified a new site that is crucial for PINCH-1 function. By expression of a PINCH-1 mutant in which the short C-terminal tail is deleted in PINCH-1 knockdown cells, we have shown that it is dispensable for PINCH-1-mediated regulation of cell spreading and survival signaling. The functions of the C-terminal tail are 2-fold. It facilitates PINCH-1 localization to cell-ECM adhesions. Although there is no doubt that this is important for the functions of PINCH-1, the C-terminal tail apparently is also required after PINCH-1 localizes to cell-ECM adhesions, as replacing it with a sequence derived from the C terminus of PINCH-2 restores the PINCH-1 localization to cell-ECM adhesion but not its functions in cell spreading and survival signaling. The identification of the C-terminal tail as a key site that regulates both the localization and the post-localization signaling of PINCH-1 should facilitate future studies aimed at identifying additional PINCH-1 binding partners involved in these processes.

Although the PINCH-1 C-terminal tail participates in cell shape modulation and survival signaling, it is not required for the maintenance of the ILK protein level. Thus, among the three PINCH-1 functional sites (LIM1, LIM4, and the C-terminal tail), only LIM1 is involved in the maintenance of the ILK level. Cell survival signaling involves at least two sites (i.e. LIM1 and the C-terminal tail). PINCH-1-mediated modulation of cell morphological change appears to be most demanding among the three PINCH-1-mediated processes. It requires all three PINCH-1 functional sites. The studies described in this paper have firmly established the functional importance and specificity of each of these three sites. Recent studies have demonstrated that Ras suppressor 1 (RSU-1) interacts with the C-terminal region of PINCH-1 (11, 12). The PINCH-1-RSU-1 interaction, like the PINCH-1-ILK and ILK-parvin interactions (for review, see Refs. 2–4, 22, and 23), is evolutionarily conserved and functionally important for embryonic development (11). Interestingly, Dougherty et al. (12) have shown that PINCH-2 does not interact with RSU-1.

Bock-Marquette et al. (18) recently reported that PINCH-1 and ILK function as key regulators of cardiac cell migration, survival and repair. Furthermore, they have shown that PINCH-1, through its C-terminal region, interacts with thymosin β4 (18). Although the specific thymosin β4-binding site within the PINCH-1 C-terminal region remains to be determined, PINCH-1 could function in cardiomyocytes by linking thymosin β4 with ILK and other binding partners through its distinct binding sites and, hence, promotes cardiac cell migration, survival, and consequently, cardiac repair after myocardial infarction (18). Protein-protein interactions provide the foundation for control of cellular architecture and signal transduction (43). With the arrival of the post-genomics era, it has become increasingly clear that a large number of key protein-protein interactions are mediated by a relatively small number of scaffolding proteins, which through its multiple protein-binding motifs, provide a hub or platform for protein interactions. The studies presented in this paper together with previous studies suggest that PINCH-1, through interactions mediated by LIM1, LIM4, the C-terminal tail, and perhaps other yet to be identified sites, functions as one of the key protein binding platforms at the cell-ECM adhesions controlling cell shape modulation and survival.

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