Hic-5 is a focal adhesion LIM protein serving as a scaffold in integrin signaling. The protein comprises four LD domains in its N-terminal half and four LIM domains in its C-terminal half with a nuclear export signal in LD3 and is shuttled between the cytoplasmic and nuclear compartments. In this study, immunoprecipitation and in vitro cross-linking experiments showed that Hic-5 homo-oligomerized through its most C-terminal LIM domain, LIM4. Strikingly, paxillin, the protein most homologous to Hic-5, did not show this capability. Gel filtration analysis also revealed that Hic-5 differs from paxillin in that it has multiple forms in the cellular environment, and Hic-5 but not paxillin was capable of hetero-oligomerization with a LIM-only protein, PINCH, another molecular scaffold at focal adhesions. The fourth LIM domain of Hic-5 and the fifth LIM domain region of PINCH constituted the interface for the interaction. The complex included integrin-linked kinase, a binding partner of PINCH, which also interacted with Hic-5 through the region encompassing the pleckstrin homology-like domain and LIM domains of Hic-5. Of note, Hic-5 marginally affected the subcellular distribution of PINCH but directed its shuttling between the cytoplasmic and nuclear compartments in the presence of integrin-linked kinase. Uncoupling of the two signaling platforms of Hic-5 and PINCH through interference with the hetero-oligomerization resulted in impairment of cellular growth. Hic-5 is, thus, a molecular scaffold with the potential to dock with another scaffold through the LIM domain, organizing a mobile supramolecular unit and coordinating the adhesion signal with cellular activities in the two compartments.

Protein-protein interaction provides the fundamental and structural molecular framework for biological systems, organizing a diverse array of cellular activities. According to genome-wide analyses, it is likely that biological networks make use of a restricted number of modular protein-binding domains, such as Src homology 2 (SH2)2, SH3, WD, PDZ, and the ankyrin repeat in a cassette-like fashion to generate complexity. In such biological systems, the so-called scaffolding protein, which possesses multiple modular protein-binding domains, is thought to play an essential role in integrating diverse cellular pathways. Like a hub, it mediates multiple protein-protein interactions simultaneously, thereby assembling multicomponent complexes as well as directing targets to discrete subcellular locations and modulating their activities.

The LIM domain, whose designation is derived from the first letter of the founders LIN-11, Isl1, and MEC-3, is one of the modular protein-binding domains found in numerous eukaryotic proteins, consisting of a conserved cysteine/histidine-rich sequence coordinating two zinc ions that organize a double zinc finger structure essential for LIM domain function. As described in the extensive review by Kadrmas et al. (1), 135 LIM-encoding sequences are predicted to exist within as many as 58 genes in the human genome. In comparison, sequences for two other protein-binding domains, SH2 and -3, number 115 and 253, respectively. The LIM sequences are often connected with sequences encoding heterologous domains including the homeodomain, catalytic domains, and cytoskeleton-binding domains or other protein-binding modules, such as SH3, PDZ, and LD motifs. The combinations of LIM domains with a variety of these heterologous domains, characterizing individual families of LIM proteins, are the molecular basis for their roles in processes ranging from gene expression and cell adhesion to signal transduction. Among the LIM families, there are some consisting of LIM domains and other modular protein-binding domains and known to function as scaffolding proteins for cellular signaling.

Hic (hydrogen peroxide-inducible clone)-5 is one such scaffolding LIM protein belonging to the paxillin family, consisting of four LIM domains at its N terminus and four LIM domains at its C terminus (2–4). It is primarily located at focal adhesions, serving as a scaffold of integrin signaling through interaction with multiple structural and signaling molecules, such as focal adhesion kinase, PYK2/Cakβ, protein-tyrosine phosphatase PEST, vinculin, the Arf-Gap family protein GIT1, Csk, and PKL (4, 5). However, we recently found that Hic-5 shuttles between the nuclear and cytoplasmic compartments and identified the nuclear export signal (NES) overlapping with one of the LIM motifs at its N terminus (6). Besides Hic-5, an increasing number of the LIM domain proteins have been shown to shuttle between the two compartments through a NES, and these proteins must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: SH2, Src homology 2; SH3, Src homology 3; NES, nuclear export signal; ILK, integrin-linked kinase; HA, hemagglutinin; NLS, nuclear localization signal; aa, amino acids; BrdUrd, bromodeoxyuridine; LMB, leptomycin B.
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teins are expected to have a role in coordinating cellular activities in both compartments (e.g. the cell adhesion status sensed at focal adhesions with gene transcription in the nucleus) (see Ref. 1 and references therein). The NES of Hic-5 is unique in demonstrating oxidant-sensitivity dependent on two particular cysteine residues, which causes the protein to be distributed in the nucleus under oxidative conditions. Thus, another function of Hic-5 has now emerged, that of a molecular scaffold specialized for mediating the redox signaling to the nucleus (6).

In this study, we found that Hic-5 was capable of homo- and hetero-oligomerization, depending on its particular LIM domain, which potentially extends its role as a molecular scaffold quantitatively or qualitatively. Previously, we found that a LIM protein, CRP (cysteine-rich protein), bound Hic-5 (7). As a new partner in the hetero-oligomerization, PINCH (particularly interesting new cysteine and histidine-rich protein), which is another focal adhesion LIM protein that serves as a scaffold coupling integrin signals with those of growth factors (8), was identified here. In addition, the hetero-oligomer that formed between Hic-5 and PINCH was suggested to include integrin-linked kinase (ILK) and implicated in the growth control of cells. Most interestingly, the interaction with Hic-5 directed PINCH and CRP, which are primarily located at actin-based structures in the cytoplasm, to move in and out of the nucleus, dependent on the NES of Hic-5. These findings provided evidence of a protein-protein interaction through the LIM module organizing a mobile unit for proteins with dual functions in the cytoplasm and the nucleus to shuttle between the two compartments.

MATERIALS AND METHODS

Cell Culture and Reagents—Cells were maintained in Dulbecco’s modified Eagle’s medium (COS-7, 293FT, MC3T3, and NIH3T3) supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin at 37 °C in an atmosphere of 5% CO₂ in air.

Primary embryonic fibroblasts were prepared from pregnant mice on day 15 of gestation as described previously (9) and cultured in Eagle’s minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin under the same conditions as above. Once confluent, they were passaged at a dilution of 1:8 and used prior to the 10th passage. Leptomycin B was purchased from LC Laboratories (Woburn, MA).

Expression Vectors and Transfection—Mammalian expression vectors were all based on pCG-N-BL (HA-tagged) (6) and pcDNA3 (FLAG-tagged) (Invitrogen) and are prefixed with pCG- and FLAG-, respectively. The vectors for HA-tagged wild-type proteins (Hic-5, paxillin, PINCH-1, and ILK) used in this study were pCG-LD1mhic-5(WT) (10), pCG-pax (paxillin) (10), and pCG-PINCH and -ILK generated with a PCR-based method by amplifying the coding sequences using Marathon-ready cDNA of mouse 17-day embryos (BD Biosciences Clontech) as a template and inserting them into the vector. Those for FLAG-tagged versions were FLAG-LD1mhic-5(WT), FLAG-pax (paxillin) (11), and FLAG-PINCH and -ILK constructed by the same PCR-based method using the pCG-based constructs as templates. FLAG-CRP1, -2, and -3 were created by the PCR-based method with Marathon-ready cDNA of mouse 17-day embryos as a template.

The expression vectors for a set of N-terminal truncated mutants of Hic-5 were as follows (6): pCG-hhic-5(WT), pCG-delLD1–2hic-5 (ΔLD1,2), pCG-delLD3–4hhic-5 (ΔLD3,4), pCG-delLD3hic-5 (ΔLD3), and pCG-hhicLIM (LIM). Those for LIM domain mutants of Hic-5 (6) were pCG-LD1mhic-5(WT), pCG-LD1mhic-mL1/mL2/mL3 (mL1M1, -2, and -3, respectively), pCG-LD1mhic/delL4 (ΔLIM4), and pCG-LD1mhic/delL2,3,4 (ΔLIM2–4). The vector for the NES mutant (H hic-5) of pCG-mhic-5 in which an amino acid in LD3 was substituted to disrupt the NES was described previously (6). For the nuclear targeted Hic-5, the PCR-amplified nuclear localization signal (NLS) from the SV40 large T antigen was inserted into the vectors for the wild type (6). pCG-only LIM4 (only LIM4) was created by inserting the PCR-amplified LIM4 portion from amino acid 397 to 461 of LD1mhic-5 (10) into the vector.

The LIM-truncated mutants of PINCH-1 were expressed from the vectors on the basis of FLAG-PINC, including cDNA fragments for the respective portions of PICNCH-1 amplified by PCR instead of the full-length version. The amplified fragments were LIM1–2 (amino acids (aa) 2–140), LIM2–4 (aa 81–262), LIM4–5 (aa 203–337), LIM1–3 (aa 1–207), and ΔLIM5 (aa 2–262). For a series of FLAG-tagged mutants of ILK, the PCR-amplified DNA fragments were cloned into the vector. The fragments for each mutant encoded amino acid residues as follows: Δ kinase (aa 1–167), Δ paxillin-binding subdomain (aa 1–375), and Δ ANK (aa 167–452). ILK273 was created by cutting out enzymatically residues 274–452 of the full-length ILK from FLAG-ILK. To generate E359K, a point mutation was introduced into FLAG-ILK using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with mutant primers according to the manufacturer’s instructions.

Retroviral expression vectors were based on the vector PMXs-IG (12). The coding region containing the FLAG tag at the N terminus was excised from the FLAG-tagged pcDNA3 constructs and inserted into the vector. Infection was carried out as described before (7).

All PCR amplifications were carried out with pfu grade polymerase, and the products were verified by DNA sequencing.

The expression vectors were introduced into cells using the conventional calcium phosphate precipitation method (293FT) or with TransIT-LT1 (PanVera, Madison, WI) in the case of COS-7.

Antibodies, Immunoprecipitation, Western Blotting and Immunocytochemistry—The monoclonal and polyclonal anti-HA antibodies (12CA5 and Y-11) and the monoclonal anti-FLAG antibody (M2) were described previously (6). Monoclonal antibodies to Hic-5, paxillin, PINCH, and ILK were purchased from BD Biosciences. The procedures used for immunoprecipitation, Western blotting, and immunocytochemistry were essentially the same as described before (11).

Preparation of Recombinant Protein—The procedure used to prepare proteins was essentially the same as described previously (13). For full-length Hic-5, the BL21 strain of Escherichia
coli transformed with the pET16b vector (Novagen), including the coding sequence of an LD1-null form of mouse hic-5 (2), pET16b-mhic-5, was induced to produce the protein by the addition of 1 mM isopropyl-d-thiogalactopyranoside for 3 h at 30 °C. The cells were lysed, and the insoluble fraction was collected by centrifugation followed by washing. After solubilizing in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10 mM dithiothreitol) containing 8 M urea, the fraction was affinity-purified with a HisTrap Kit (Amersham Biosciences), and the protein was renatured by successive dialysis against lysis buffer containing 4, 2, 1, and finally 0 M urea for several hours at 4 °C. The renatured condition was compatible with the DNA binding assay (13), and the protein was used for cross-linking analysis.

For expression of GST-Hic-5 LIM domain fusion proteins, GST-LIM1–4 (containing all four LIM domains) and GST-ΔLIM4 (containing LIM1 to -3 domains), E. coli strain BL21 was transformed with the pGEX-5X-1-based expression vectors described previously (13) and induced with 0.1 mM isopropyl-d-thiogalactopyranoside for 24 h at 25 °C in the presence of 1 mM ZnCl₂. Extracts were prepared using BugBuster (Novagen) supplemented with 1 mM ZnCl₂ and protease inhibitor mixture (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and used as sources of the fusion proteins. The fusion proteins were immobilized on glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer’s instructions and used for pull-down assays or eluted from the beads in elution buffer (20 mM GSH, 100 mM Tris, pH 8.0, 120 mM NaCl, 1 mM ZnCl₂, and 0.5% Nonidet P-40) and used for cross-linking analyses.

Cross-linking Analysis—The protein was incubated in the presence or absence of 0.001–0.01% glutaraldehyde in the lysis buffer (full-length) or the elution buffer (GST fusion) for 30 min at 37 °C. The reaction was terminated by adding SDS-PAGE sample buffer and further analyzed by Western blotting.

Gel Filtration Chromatography—Cells were solubilized in buffer (0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 6.8, and protease inhibitor mixture (Wako Pure Chemical Industries, Ltd., Osaka, Japan)) and, after centrifugation, fractionated by gel filtration through a HiPrep 16/60 Sephacryl HR (fraction range, 1.33-ml fractions). The collected fractions were analyzed by Western blotting. Molecular weight standards of protein (MW-GF1000; Sigma) were analyzed under the same chromatographic conditions.

Immunoelectron Microscopy—Portions of mouse aorta tissue were incubated for more then 3 days in cold 1.84 M sucrose in 0.1 M phosphate buffer containing 20% polyvinyl pyrrolidone. After being cut into small pieces, they were rapidly frozen in liquid nitrogen at −196 °C. Frozen ultrathin sections (100 nm) were cut with a Lica Ultracut R (Vienna, Austria). The sections were picked up on a Formvar-carbon-coated nickel grid, incubated with 2% gelatin in phosphate-buffered saline containing 10 mM glycine, and allowed to react with 1:500 diluted anti-Hic-5 monoclonal antibody (BD Transduction Laboratories) or antivinculin monoclonal antibody (Sigma) overnight. The sections were washed five times with glycine/phosphate-buffered saline containing 0.5% gelatin and then incubated for 2 h with 10-nm colloidal gold-labeled rabbit anti-mouse IgG antibody (EY Laboratories, Inc.). After being washed again, the sections were postfixed in 0.1% glutaraldehyde, stained with 2% uranyl acetate, embedded in polyvinyl sacohol, and observed with a Joel JEM-1200 EX II electron microscope (Joel, Tokyo, Japan).

In Vitro Translation and Pull-down Assay—In vitro translation was performed from the pcDNA3 plasmids carrying a full-length PINCH and ILK preceded by a T7 promoter with a TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI) in the presence of Transcend biotinylated tRNA (Promega, Madison, WI) in a final volume of 50 µl. For the pull-down assay, 15 µl of in vitro translated product was incubated with GST or GST-LIM1–4 beads (~2 µg of protein) in 500 µl of binding buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, and 0.1% bovine serum albumin) for 90 min at 4 °C. After a wash with the binding buffer and then with phosphate-buff ered saline, bound PINCH and ILK proteins were resolved by SDS-PAGE, blotted on a membrane, and detected with streptavidin-horseradish peroxidase (Amersham Biosciences).

Cell Growth and BrdUrd Incorporation—The retrovirally infected cells were seeded at 2 × 10⁵ cells in 35-mm dishes. The total cell number was counted every day with a hemocytometer until 5 days after seeding. Cell viability was assessed by trypsin blue staining.

For the incorporation of BrdUrd, the infected cells were serum-starved for 36 h and then stimulated by replacing the...
medium with fresh medium containing 10% fetal bovine serum and BrdUrd (1 μg/ml). At 16 h after the stimulation, cells were fixed with 70% ethanol for 30 min at room temperature and then processed for immunocytochemistry with a Cell Proliferation Kit (Amersham Biosciences).

RESULTS

Self-association of Hic-5—We first addressed the self-association of Hic-5 proteins labeled with two different tags in immunoprecipitation experiments. As shown in Fig. 1A, we co-expressed HA- and FLAG-tagged Hic-5 in COS-7 cells and immunoprecipitated the HA-tagged Hic-5 with the antibody to the tag and then analyzed the precipitates by Western blotting with the antibody to FLAG. The results clearly showed the presence of FLAG-tagged Hic-5 in the immunocomplex precipitated with the antibody to HA, indicating the ability of Hic-5 to self-associate in cells (Fig. 1A, lane 2H). The population making the complex was estimated to account for less than 10% of the total population based on the intensity of bands. However, this value appeared to be underestimated, since the population in complexes with the same tagged proteins was not represented. Surprisingly, a similar experiment with paxillin, the protein most homologous to Hic-5, revealed neither self-association nor an association with Hic-5 (Fig. 1A, lanes 3H and 4H), underlining the specificity of the self-association of Hic-5 and making it unlikely that the association resulted from overexpression of the protein. We also carried out the experiment with proteins engineered to be localized to the nucleus by adding a nuclear localization signal (NLS). Given that Hic-5 shuttled between the cytoplasm and nucleus in cells, a fraction of Hic-5 was expected to be present in the nucleus (6). As demonstrated in Fig. 1B, the NLS plus form of Hic-5 self-associated efficiently, even more than the wild type, suggesting the self-association of Hic-5 both in the cytoplasm and in the nucleus. In C3H10T1/2 mouse fibroblasts, a similar self-association was observed (data not shown).
Determination of the Domains Responsible for Self-association—To determine the domain mediating the self-association, a set of mutated and deleted forms of Hic-5 was tested for their association with full-length Hic-5 using the same experimental procedure as in Fig. 1. The results shown in Fig. 2, A and B, suggested that the most C-terminal LIM domain, LIM4, was critically involved in the self-association of Hic-5. Furthermore, when isolated, the LIM4 domain had the ability to bind the full-length Hic-5, indicating that LIM4 was not only necessary but also sufficient for the association (Fig. 2C). N-terminal LD domains 1 and 2 also appeared to play a role, although not an essential one, in the association, since their deletion lessened the degree of association (Fig. 1A, lane 1F versus lane 2F). As summarized in Fig. 2D, including observations that are not described here, the results demonstrated the capability of Hic-5 to self-associate through LIM4 in cultured mammalian cells. Of note, despite its overall structural similarity to Hic-5 and high homology in the LIM4 domain at the amino acid level with Hic-5 (4), paxillin showed no ability to self-associate.

In Vitro and In Vivo Analysis of Self-association—In further experiments, we investigated the self-association of Hic-5 in vitro. First, to test whether Hic-5 can bind itself directly or not, we carried out cross-linking experiments with affinity-purified recombinant proteins. Full-length (Fig. 3A) and GST-Hic-5 LIM domain fusion proteins (Fig. 3B) produced in E. coli were subjected to cross-linking with glutaraldehyde, and the cross-linked products were analyzed by SDS-PAGE followed by Western blotting or silver staining. As seen in Fig. 3A, along with a monomer of about 50 kDa, multimeric species with molecular weights corresponding to the dimer, trimer, and higher multimers. The open arrowheads show an unrelated E. coli protein. The amount of the protein cross-linked was 2.5 μg/50-μl reaction (A), 2 μg (lanes 1 and 2), 7 μg (lanes 3 and 4), and 13 μg (lane 5) of protein/50-μl reaction were cross-linked. C, cells were solubilized in the buffer, and after being cleared by centrifugation, the lysate was fractionated by gel filtration chromatography and analyzed by Western blotting with the antibodies to HA tag, Hic-5, and paxillin as described under "Materials and Methods." For exogenous Hic-5, MEF/Tet-Off cells that were induced to express HA-tagged LD1 m hic-5 by removal of doxycyclin for 24 h were used, and for endogenous Hic-5, primary mouse embryonic fibroblasts were used. The arrowheads show the four peaks identifiable in the elution profile. D, an ultrathin section of a mouse aorta was incubated with the anti-Hic-5 (Hic-5) or anti-vinculin (vinculin) monoclonal antibody, labeled with the 10-nm gold-labeled secondary antibody, and examined under an electron microscope. An image of a part of a smooth muscle cell is shown. Scale bar, 100 nm. E, the expression vectors for the HA-tagged Hic-5 (WT) and for the LIM mutants (mLIM1–3 and mLIM4) were introduced into C3H10T1/2, and 24 h later, the cells were fixed and processed for immunocytochemistry as described under "Materials and Methods" with the anti-HA antibody (12CA5). F-actin was visualized with fluorescein isothiocyanate-conjugated phalloidin labeling, and the nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI).
molecular masses expected for the dimer, trimer, tetramer, and higher multimeric forms were detected with an increase in the concentration of glutaraldehyde, suggesting that the Hic-5 protein is capable of homo-oligomerization by itself in vitro. Similarly, consistent with the result of domain mapping (Fig. 2), wild-type LIM domains consisting of LIM1–4 fused with GST (GST-LIM1–4) oligomerized, but not those lacking LIM4 (GST-ΔLIM4), even at a more than 6-fold higher concentration than the wild type, supporting homo-oligomerization of Hic-5 mediated through LIM4.

Second, to obtain insight into the state of Hic-5 in the cellular environment, we analyzed whole cell lysate containing Hic-5 that was endogenously or exogenously expressed through gel filtration chromatography. As the exogenous protein, HA-tagged Hic-5, induced in MEF/Tet-Off cells harboring tetracycline-responsive hic-5 cDNA (6) by removing doxycyclin from the culture medium, was examined. As a source of the endogenous protein, we used primary mouse embryonic fibroblasts that showed relatively high levels of expression of Hic-5 (9). After the chromatography of these protein sources, we detected the Hic-5 eluted in fractions by Western blotting with the antibody to the HA tag for the exogenous Hic-5 and with that to Hic-5 or paxillin for each endogenous protein and obtained elution profiles as shown in Fig. 3C. According to the profiles, the exogenous and endogenous Hic-5 behaved identically, and they were reproducibly eluted as multiple species ranging from about 50 to 200 kDa with four peaks along with a broad distribution to around 400 kDa or more. The appearance of multiple peaks suggested discrete oligomeric forms of Hic-5 in cells, which resulted from either homo- or hetero-oligomerization with other LIM proteins (as below). Paxillin, on the other hand, displayed a broad distribution only in fractions of high molecular mass with no discernable evidence of multiple species (Fig. 3C, middle column), suggesting that paxillin was integrated in a large protein complex. Whereas further analysis is needed to clarify the molecular entity of each complex and determinant responsible for this unexpected difference between Hic-5 and paxillin, the ability to self-associate might explain some of the functional differences between Hic-5 and paxillin as noted in previous studies (see Ref. 6 and references therein).
Importantly, in an immunoelectron microscopic study on the subcellular distribution of Hic-5 in an in vivo setting, we observed that most immunogold particles labeling Hic-5 clustered in the cytoplasm and nucleus of smooth muscle cells in the mouse aorta, and more than half of the particles were apparently in contact with others or were spaced at a distance estimated to be about one molecular dimension (±10 nm, the diameter of a gold particle) (Fig. 3D). Similar clusters of gold particles were reported in an immunoelectron microscopy study of Alzheimer amyloid that aggregated into oligomers and accumulated in neuronal processes (14). Unlike the case of Hic-5, the particles labeling vinculin, which was colocalized with Hic-5 at focal complexes (15), were also concentrated in some areas within the cells, whereas many of them appeared to be separated from others (Fig. 3D, vinculin). This observation strongly suggested that a significant proportion of Hic-5 also homo-oligomerized under the physiological conditions in cells in vivo. Altogether, the above observations suggested that Hic-5 had the capability to oligomerize, including to homo-oligomerize.

Subcellular Distribution and Self-association of Hic-5—Hic-5 is a focal adhesion protein that is also present on actin stress fibers in the cytoplasm and in the nucleus within cells. The observation of multiple species of Hic-5 protein in cells raised the possibility that the localization to specific sites and oligomerization of the protein were interdependent. However, this was unlikely. The analysis of mutants in our previous study suggested that disruption of LIM2 and LIM3 significantly impaired the localization of Hic-5 at focal adhesions and on actin stress fibers (7). Consistent with this, the LIM domain mutants (mLIM2 and mLIM3) showed a disturbed distribution as rodlike or punctuate structures in the cytoplasm and were not localized to focal adhesions (Fig. 3E). However, these mutants retained the ability to self-associate (Fig. 2B), suggesting that the self-association occurred independently of the localization to the actin-related cytoarchitecture. Likewise, the distribution of Hic-5 to the actin-related structures, including focal adhesions, was independent of self-association, since ΔLIM4, which was only able to exist as a monomer, displayed a distribution within cells apparently indistinguishable from that of the wild type, including a localization to focal adhesions. The nuclear accumulation of this mutant under oxidative conditions was also observed (6).

![Diagram of Hic-5 and PINCH domains](image-url)

**FIGURE 5. The fourth Hic-5 and the fifth PINCH LIM domains mediate the interaction between Hic-5 and PINCH.** A, lysates of COS-7 cells expressing the FLAG-tagged PINCH with the HA-tagged Hic-5, WT (lane 5) or with a series of its LIM mutants, mLIM1–3 (lanes 1–3) and ΔLIM4 (lane 4), were immunoprecipitated with the anti-HA antibody (lane H) or control IgG (lane C) and subjected to SDS-PAGE, followed by Western blotting with the anti-HA and anti-FLAG antibodies. The wild-type Hic-5 and its variants used in the experiments and a summary of the results are schematically represented. B, the HA-tagged Hic-5 was expressed in COS-7 cells alone (lanes 5 and 6) or with the FLAG-tagged PINCH (WT, lanes 4 and 8) or a series of its deletion mutants, LIM1–2 (lane 1), LIM2–4 (lane 2), LIM4–5 (lane 3), and ΔLIM5 (lane 7). Cell lysates were immunoprecipitated and analyzed as above. A schematic representation of the truncated mutants of PINCH used in the experiments and a summary of the results are given. The expression vectors for these proteins are described under "Materials and Methods."
Association of Hic-5 with PINCH and ILK—The finding of multiple species of Hic-5 in cells suggested the possibility that besides itself, Hic-5 oligomerized with other LIM proteins, although paxillin did not associate with Hic-5 (Fig. 1A). In fact, we recently found interaction between Hic-5 and CRP1, -2, and -3 (7). CRP is a LIM protein classified into the LIM-only group, whose members comprise almost exclusively LIM domains, together with accessory regions in some cases. Here we focused on PINCH, another of the LIM-only proteins, consisting primarily of a tandem array of five LIM domains and a short C-terminal tail. Similar to Hic-5, PINCH, widely expressed at focal adhesions, has been shown to interact with signaling molecules through the LIM domains, thereby serving as a potential molecular platform coupling and uncoupling signaling pathways to coordinate cellular processes at focal adhesions (8). The binding partners of PINCH identified so far include ILK and Nck-2, a SH2/SH3-containing adaptor protein (8). In Fig. 4, A and B, PINCH-1 and either Hic-5 or paxillin, which were tagged differently, were co-expressed in COS-7 cells and subjected to immunoprecipitation experiments as above. PINCH-1 was co-precipitated with Hic-5 but not with paxillin, and Hic-5 in turn was co-precipitated with PINCH-1 (Fig. 4A) and PINCH-2 (data not shown), suggesting that Hic-5 heterodimerized with PINCH. The intensity of the bands suggested that the efficiency of the heterodimerization was almost equivalent to that of homo-oligomerization. PINCH-1 is herein referred to simply as PINCH and was subjected to further analysis. FHL2 (four- and a half LIM 2), a member of the same LIM-only protein family, did not interact with Hic-5 (data not shown), suggesting selectivity in the binding of Hic-5 to the family members.

In addition to the interaction of Hic-5 with PINCH, we also found that Hic-5 bound ILK, a binding partner of PINCH. In a cross-immunoprecipitation experiment, Hic-5 and ILK were co-precipitated (Fig. 4B). The result was comparable with that with ILK and paxillin, which were previously shown to interact with each other (16) (Fig. 4B). Because ILK was shown to bind PINCH (8), these three proteins were consequently assumed to interact with each other in three ways to form Hic-5-PINCH, Hic-5-ILK, and PINCH-ILK. The possibility of a trimeric complex was also suggested by sequential immunoprecipitation (data not shown).

Determination of Domains Mediating the Hic-5-PINCH and Hic-5-ILK Interactions—The domains mediating the Hic-5-PINCH and Hic-5-ILK interactions were determined by utilizing a series of mutants in immunoprecipitation experiments as above. First, for the binding of Hic-5 to PINCH, chimeras of the LIM domains of Hic-5 and the central region of ILK mediate the interaction between Hic-5 and ILK. A, lysates of COS-7 cells expressing the FLAG-tagged ILK alone (lane 1) or with the HA-tagged Hic-5 (lane 4) and its LIM-truncated mutants, ΔLIM2–4 (lane 2) and ΔLIM4 (lane 3), were immunoprecipitated with the anti-FLAG antibody (lanes F) or control IgG (lanes C) and subjected to SDS-PAGE followed by Western blotting with the antibodies to FLAG and HA. A schematic representation of the series of truncated mutants of Hic-5 used in the experiments and a summary of the results are shown. B, the expression vector for the HA-tagged Hic-5 was introduced into 293FT cells with an empty vector (lane 1) or with the vectors for the FLAG-tagged wild-type ILK (lane 2) and its variants, Δkinase (lane 3), ΔANK (lane 4), Δpaxillin-binding subdomain (ΔPBS) (lane 5), E359K (lane 6), and 273 (lane 7). Cell lysates were immunoprecipitated with the anti-HA antibody (lanes H) or control IgG (lanes C) and analyzed as above. A schematic representation of ILK and its variants used in the experiments and a summary of the results are shown. A star indicates the lysine residue at position 359 substituted for glutamic acid. The expression vectors for these proteins are described in "Materials and Methods."
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was determined (Fig. 5B) and is schematically represented. The results pointed out the importance of the C-terminal end containing domain LIM5 of PINCH for its binding to Hic-5.

Likewise, the domains of Hic-5 responsible for the binding to ILK and those of ILK responsible for the binding to Hic-5 were studied (see Fig. 6, A and B, respectively). The binding of Hic-5 to ILK was remarkably diminished by eliminating LIM4 of Hic-5 and completely disappeared with further deletion of LIM2 and -3 (Fig. 6A, lanes 3F and 2F), whereas the deletion of LD domains in the N-terminal half did not affect the interaction (data not shown). On the other hand, ILK was found to bind Hic-5 through its C-terminal half, the so-called kinase domain, containing a pleckstrin homology-like domain and a paxillin-binding subdomain, whereas it bound LIM1 of PINCH through the N-terminal four ANK repeats (17). Further study narrowed the region of ILK required for binding to Hic-5 to around the pleckstrin homology domain (Fig. 6B). In summary, the interaction between Hic-5 and ILK occurred through the LIM domains of the C-terminal half of Hic-5 and a central part of ILK encompassing the pleckstrin homology domain (Fig. 6, A and B). The interaction between paxillin and ILK, on the other hand, was reported to be mediated through the N-terminal LD1 domain of paxillin and the paxillin-binding subdomain in the C-terminal of ILK (16), suggesting that the two complexes ILK-Hic-5 and ILK-paxillin were assembled differently through discrete domains of ILK with or without the additional protein, PINCH, included. Consistent with this notion, the point mutation of Glu to Lys at amino acid 359 of ILK, which was believed to affect both the kinase activity and binding to paxillin (18), had no influence on the binding to Hic-5 (Fig. 6B).

Interactions among the Endogenous Proteins and in Vitro—
Not only the exogenously overexpressed proteins but also the endogenous proteins were found to make complexes. The immunoprecipitation of cell lysate prepared from primary mouse embryonic fibroblasts with the antibody to Hic-5 and ILK demonstrated that Hic-5 interacted with ILK and PINCH (Fig. 7A, IP: Hic-5) and that ILK interacted with Hic-5 and PINCH (Fig. 7A, IP: ILK).

Based on the results in Figs. 4–7, we concluded that Hic-5, PINCH, and ILK interacted with each other as schematically illustrated in Fig. 7B. It should be pointed out that the domains mediating each interaction were specific to the respective interaction, since Hic-5 bound the central region of ILK, whereas PINCH bound the N-terminal ANK1, making it unlikely that the binding of Hic-5 to ILK was mediated by PINCH. Otherwise, the binding of Hic-5 to ILK would be dependent on ANK1 of ILK, the binding domain for PINCH. Similarly, the interaction between Hic-5 and PINCH was presumably direct. In fact, a pull-down assay in which in vitro translated PINCH and ILK were precipitated with beads immobilizing GST-Hic-5 LIM domain fusion protein (GST-LIM1-4) supported the possibility that the interactions between Hic-5 and ILK and between Hic-5 and PINCH were direct (Fig. 7C).

Roles of ILK and Hic-5 in the Regulation of the Subcellular Distribution of PINCH—Hic-5, PINCH, and ILK are all described as focal adhesion proteins localized to cell matrix adhesion sites. Previous studies suggested that ILK and

Hic-5 and paxillin were tested. Since paxillin did not interact with PINCH, the chimeric protein whose C-terminal half was from paxillin (Hic/pax) but not the reciprocal chimera (Pax/Hic) lost the ability to interact (data not shown), suggesting the importance of the C-terminal half containing the four LIM domains of Hic-5 for the interaction with PINCH. To assess the contribution of each LIM domain, we disrupted them individually by introducing a point mutation (LIM1–3) or deleted LIM4 and found that these manipulations resulted in decreased binding of Hic-5 to PINCH (Fig. 5A). In particular, deletion of LIM4 of Hic-5 completely precluded the interaction (the interaction between LIM4 and PINCH was undetectable even with a longer exposure) (Fig. 5A, lane 4H), suggesting a critical role for LIM4 of Hic-5 in the association with PINCH as in the self-association of Hic-5 (Fig. 2). Through a similar experiment, the region of PINCH responsible for the interaction with Hic-5
pinch-1 mutually regulated their recruitment to focal adhesions and their stability (19, 20). For the recruitment of pinch, additional requirements were suggested, which included paxillin or a yet to be defined mechanism involving the C-terminal end of pinch (20, 21). We here examined the role of hic-5, together with that of ilk, in the regulation of the subcellular distribution of pinch. To approach this, we ectopically overexpressed pinch alone, pinch in combination with hic-5 or ilk, or the three together in cells and then visualized them using immunocytochemistry with the antibodies to tags. When expressed alone, pinch was distributed throughout the cell, including the nucleus, under our experimental conditions (Fig. 8C). In previous studies, several conflicting observations were made on the subcellular distribution of pinch. For example, Zhang et al. (21) and Xu et al. (20) reported a clear localization of pinch fused with green fluorescent protein to focal adhesions in C2C12. However, another study reported the cytoplasmic, perinuclear, and nuclear localization of endogenous pinch in schwann cells (22), implying an indecisive distribution of pinch in cells. On the other hand, hic-5 and ilk were found at focal adhesions and in the cytoplasm (Fig. 8, A and B), consistent with previous studies (3, 23), suggesting that hic-5 and ilk intrinsically harbored a sequence causing them to be recruited to focal adhesions on their own, whereas pinch required tethering by others for its distribution to discrete sites in the cell.

When pinch was co-expressed with ilk but not with hic-5, its disrupted distribution was improved to some extent, and the co-localization of pinch with ilk at discrete sites at the cell periphery was discernable, although most pinch was present in the cytoplasm (Fig. 8, I and J and merge in the same row). The prominent change induced by ilk was the exclusion of pinch from the nucleus. Upon co-expression of the three proteins, pinch was co-localized with hic-5 and ilk at focal adhesions, similar to the endogenous protein (Fig. 8, N and P, and Fig. 9A, (−)/Pinch). These observations confirmed the previous result, pointing out the importance of ilk in the tethering of pinch to focal adhesions (21), and showed that any effect of hic-5 on the subcellular localization of pinch was marginal. Of interest, ilk could not exclude pinch from the nucleus when the C-terminal end, including LIM5 of pinch, was removed (Fig. 8Q), suggesting the involvement of the region, which overlapped with the hic-5-binding area, in the tethering of pinch by ilk. The subcellular distribution of hic-5 and ilk appeared to be autonomously regulated. Whether they were expressed with the binding partners

**FIGURE 8.** The roles of Ilk and Hic-5 in regulation of the subcellular distribution of Pinch. The HA-tagged or FLAG-tagged wild-type Hic-5, Ilk, Pinch, and the LIM5-deleted Pinch mutant (PinchΔLIMS) were expressed from the vectors as described under “Materials and Methods” either alone (Single transfection, A–D), in combinations of two of the three (Double transfection, E–J), or altogether (Triple transfection, K–Q) in C3H10T1/2 cells, and their localization was visualized by immunocytochemistry with the antibody indicated as described under “Materials and Methods.” Merged images of each combination are shown (merge).
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**FIGURE 9. The interaction with Hic-5 directs CRP and PINCH to shuttle between the cytoplasm and nucleus.** A, MC3T3 and C3H10T1/2 cells were treated with LMB (10 ng/ml) (+LMB) or mock-treated (−) for 12 h and processed for communostaining with the anti-Hic-5 and anti-PINCH antibodies. The nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI). B and C, the FLAG-tagged CRP1, -2, or -3 or PINCH was expressed in C3H10T1/2 cells in the presence or absence of the HA-tagged Hic-5 and ILK. At 24 h after transfection, cells were treated with LMB as in A, and then CRP1, -2, and -3 and PINCH were visualized by immunostaining with the anti-FLAG antibody. Representative cells showing a predominant cytoplasmic distribution (Nuclear localization (+)) or nuclear accumulation (Nuclear localization (−)) of FLAG-CRP2 and -PINCH are shown (B), the number of Nuclear localization (+) cells was scored microscopically, and the ratio was graphed (CRP1, -2, and -3 (C) and PINCH (D)) (means ± S.D. derived from a series of experiments repeated more than three times; in each of them, >50 cells were scored). PINCHLIM3Δ-TM, the LIM5-deleted variant of PINCH; H, the wild-type Hic-5; HmLD3, the NES-disrupted mutant; HmLIM4, the LIM4-deleted mutant; IL, ILK. The expression vectors are described under “Materials and Methods.” Shaded and open bars, with or without LMB treatment, respectively.

or not, their localization at focal adhesions and in the cytoplasm was basically unchanged (Fig. 8A, A, E, G, K, and M and B, F, I, L, and O).

**Hic-5-directed Nuclear-Cytoplasmic Shuttling of PINCH and CRPs—** Recently, we demonstrated that Hic-5 communicated between focal adhesions and the nucleus and identified the NES responsible for the shuttling (6). Similarly, endogenous PINCH was characterized as a shuttling protein previously (22). Consistent with these findings, we observed a modest increase in the nuclear staining of endogenous PINCH in response to leptomycin B (LMB), an inhibitor of the nuclear export signal, like that of Hic-5, in two murine mesenchymal cell lines (Fig. 9A). These findings together with the hetero-oligomerization of PINCH with Hic-5 as described above lead us to assume that Hic-5 was involved in the shuttling of PINCH, although the subcellular distribution of PINCH, at least in a stationary state, was affected little by Hic-5. We explored this possibility by expressing PINCH with or without Hic-5 and ILK in cells and examining their localization in the presence of LMB. The expressed proteins were immunolabeled with the antibody to the tag, and the frequency of their nuclear localization as shown in Fig. 9B, (+), was quantitatively evaluated among the cell population under a microscope as described under “Materials and Methods.” When PINCH was overexpressed alone, it distributed in the cytoplasm and nucleus at an almost equal rate (Fig. 8C), and unlike for the endogenous PINCH, this pattern was unaltered by LMB treatment (Fig. 9C, −/−/PINCH). Then we tried to reconstitute the responsiveness to LMB of PINCH by introducing Hic-5 or ILK into cells with PINCH. Under the expression of either of the two, however, the localization of PINCH remained insensitive to LMB treatment, although the ILK expression significantly reduced the distribution of PINCH in the nucleus as observed above (Fig. 9C, H/−/PINCH and −/IL/−/PINCH). Of interest, when both Hic-5 and ILK were co-expressed with PINCH, PINCH exhibited a marked accumulation in the nucleus in response to LMB treatment, suggesting that it was endowed with the ability to communicate between the cytoplasm and nucleus through a CRM1-dependent NES function (Fig. 9C, H/IL/−/PINCH). Importantly, the NES mutant of Hic-5 (HmLD3) was incompetent in inducing the shuttling of PINCH even in the presence of ILK, suggesting a crucial role for the NES of Hic-5 in inducing the shuttling of PINCH (Fig. 9C, HmLIM4/ILK/−/PINCH). The results with each binding-defective mutant of Hic-5 (HmLIM4) and PINCH (PINCHLIM3Δ) indicated that the interaction between Hic-5 and PINCH was a prerequisite for the shuttling of PINCH (Fig. 9C, HmLIM4/ILK/−/PINCH and H/IL/−/PINCH). These observations suggested that, through binding to Hic-5, PINCH was driven in and out of the nucleus in a manner dependent on the Hic-5 NES. ILK was also involved in the regulation somehow (Fig. 9C, H/−/PINCH versus H/IL/−/PINCH), being localized invariably in the cytoplasm under the experimental conditions irrespective of the co-expression of the partners and LMB treatment (data not shown). Hic-5 except for the NES mutant HmLD3 was accumulated in the nucleus independently of the co-expression of PINCH and ILK in nearly 70–80% of the cells upon LMB treatment as reported in our previous study (6). Likewise, we observed that Hic-5 facilitated the shuttling of CRP proteins, the other partners of Hic-5 in hetero-oligomerization (Fig. 9D) (7). When co-expressed with Hic-5, the accumulation of CRP1 and -2 in the nucleus increased in the cell population, albeit in one-third of the cells at most, in response to LMB, whereas that of CRP3 was minimally affected.

From these findings, we proposed a new role for Hic-5 as an organizer of the nuclear-cytoplasmic shuttling of the LIM protein complex based on the hetero-oligomerization of Hic-5 with partners that were primarily immobile by themselves, such as PINCH and CRP1 and -2.
Involvement of the Hic-5-PINCH Complex in Cell Growth Control—Both Hic-5 and PINCH interacting with multiple signaling molecules have been assumed to serve as platforms coupling and uncoupling signaling cascades. Thus, the interaction of Hic-5 with PINCH that leads to the docking of the two signaling platforms hypothetically enables physical and functional communications between the signaling molecules on the individual platforms. To address the biological significance of the formation of such a supramolecular unit, we here investigated the involvement of the Hic-5-PINCH complex in cell growth control. To this end, we first attempted the depletion of Hic-5 with siRNA, which could effectively decrease the overall expression level of Hic-5. However, immunocytochemistry showed a substantial signal for Hic-5 remaining at focal adhesions, implying a selective knockdown except at focal adhesions. Then we took advantage of the overexpression of the mutants of Hic-5 (Hic^ΔLIM4) and PINCH (PINCH^ΔLIM5) that were unable to interact with each other, thereby breaking down the communication of the signaling molecules between the platforms. In the experiment that followed, the above mutants were introduced into NIH3T3 cells by a retrovirus-mediated method, and cell growth was assessed by measuring doubling time and the incorporation of BrdUrd. Upon the introduction of either mutant, cell growth was suppressed. When the cells were infected with a retroviral construct encoding the PINCH binding-defective Hic-5 (Hic^ΔLIM4) or its nuclear localizing version (NLS-Hic^ΔLIM4), the doubling time was extended about 1.7-fold, and BrdUrd incorporation was reduced to at most 60% compared with the controls infected with the empty vector or wild-type Hic-5 construct (Fig. 10, A and B). The expression level of the introduced protein was comparable between the wild type (Hic-5) and the mutant (Hic^ΔLIM4), whereas that of the nucleus-localizing version of the mutant (NLS-Hic^ΔLIM4) was lower (Fig. 10 C). Nevertheless, NLS-Hic^ΔLIM4 displayed a more effective suppression of cell growth, suggesting that the action of the complex occurred in the nucleus. Similarly, the Hic-5 binding-defective mutant of PINCH (PINCH^ΔLIM5) expressed at almost equivalent levels to the wild type signifi-
DISCUSSION

Oligomerization is recognized as an extreme protein-protein interaction, in which the partner is the protein itself or a highly homologous protein, and is speculated to have evolved to amplify the same kind of signals or to facilitate collaboration among relatives. Like other protein-protein interactions, oligomerization is mediated by protein modules, representatives of which are the leucine zipper, helix-loop-helix, ankyrin, and PAS domains. Given the potential of CRPs to dimerize (24), the LIM domain is also likely to be a modular protein-binding interface mediating oligomerization.

In this study, we examined the oligomerizing ability of Hic-5 and attributed it to the most C-terminal LIM domain, LIM4. Previously, we have assigned to the individual LIM domains of Hic-5 distinctive roles, as an interface interacting with signaling molecules or as a determinant directing the localization of the protein to actin-related structures in cells (7, 25), and found that LIM4 is uniquely multifunctional. In addition to serving as an interface for interaction with itself, PINCH and ILK, it is implicated in the scaffold function of Hic-5 in the nucleus to assemble transcriptional complexes (26), in the association of Hic-5 with the nuclear matrix (27), and in the interaction with HSP27 (28), although not in the regulation of the localization to actin-based cytoarchitectures (Fig. 4B). These functions of Hic-5 mediated by LIM4 might be coupled with its homo- and hetero-oligomerization.

Basically, the LIM domain is a protein module with a broad spectrum of binding partners (1). Besides the homologous domain, it binds a number of heterologous domains, including ankyrin, SH3, homeodomain, the proline-rich domain of protein-tyrosine phosphatase PEST, LID of LDB1 protein, and other sequences with no obvious notable features. Intriguingly, no consensus sequence or structural feature seems to be shared by these binding partners. This is similar to the leucine-rich repeat and ankyrin repeats and in contrast to many classical protein-binding domains, such as SH2 and SH3, which display discrete and high affinity for particular binding partners. In addition to this diversity of binding sequences, the LIM domain has another peculiar feature in that a single LIM domain can simultaneously bind several proteins, or tandem LIM domains can synergistically bind a single partner. As noted above, this is the case for the LIM domains of Hic-5. LIM4 of Hic-5 simultaneously binds multiple partners, as mentioned above. In the binding of Hic-5 to PINCH and ILK, synergistic effects among the four LIM domains were also observed (Fig. 5A and 6A).

Regarding function, an increasing number of LIM proteins, except LIM homeodomain proteins that are exclusively nuclear and have established transcriptional roles during development, share an intriguing feature (i.e., a putative role in coordinating the actin-based cytoarchitecture with the nuclear activity on the basis of their shuttling between the cytoplasmic and nuclear compartments as discussed in the review by Kadras and Beckerle (1)). In that review, it was proposed that the presence of a LIM domain is a hallmark of proteins that can associate with both the actin cytoskeleton and the transcriptional machinery. Actually, many LIM proteins were initially identified as cytoskeleton-associated proteins, including members of the zyxin, FHL, and CRP families, and then found to communicate between the two compartments to influence gene expression. In many cases, however, the mechanism underlying their shuttling remains unclear. For example, LIM-only proteins, including CRP, do not possess a nuclear localization signal (NLS) or NES. As for PINCH, NLS- and NES-like sequences were found at its C-terminal end (22), although its actual function has not been elucidated. One way these proteins may shuttle between the compartments is to make a complex with a shuttling protein and use it as a vehicle.

Paxillin family members, including Hic-5, established as a molecular scaffold in integrin signaling at focal adhesions, were recently shown to have an NES and shuttle between the two compartments (6, 29). Another body of evidence suggested the involvement of Hic-5 in the nuclear activity like most other shuttling LIM proteins, as mentioned above (6, 26, 30–34). However, the functions characterized so far at each location are apparently independent, so it is unclear at present whether they are interrelated or not, and if they are related, it remains unsolved how they are coordinated. Accordingly, the biological meaning of the shuttling is also unexplained.

In the present study, we demonstrated most importantly that Hic-5 bound PINCH and CRP and organized the nuclear-cytoplasmic shuttling complex that assisted the shuttling of both proteins between the two compartments (Fig. 9, C and D). For CRP to function as a coactivator of transcription, its shuttling to the nucleus from the cytoplasmic actin-related cytoarchitecture is essential (35). Another partner, PINCH, is known to exist at focal adhesions. However, a recent report suggested its nuclear localization (22), although the mechanism regulating the localization and its significance have not been addressed. With regard to biological function, PINCH was suggested to play a role in the regulation of the actin cytoskeleton’s organization and integrin functions, such as controlling cell shape and survival (8, 36). In this study, we examined the biological significance of the formation of a shuttling complex between Hic-5 and PINCH and found that the docking of the two scaffolds potentially contributes to the signaling for cell growth (Fig. 10), although neither the mechanism involved nor its relation to the shuttling of the complex is known at this stage. Considering that the C-terminal end of PINCH, which is the region where Hic-5 binds, was suggested to be specifically involved in changes of cell shape and in cell survival (20), it is conceivable that the hetero-oligomerization of PINCH with Hic-5 and the shuttling to the nucleus are also involved in these functions. To understand the biological significance of the shuttling complex organized by Hic-5, more information about the role of individual components together with the regulation of the complex’s formation will be required, including information on the nuclear function of PINCH.

In the Hic-5-PINCH complex, ILK was demonstrated to be included (Figs. 4B and 7). ILK is a component of focal adhesions that directly interacts with the cytoplasmic tail of integrin β...
subunits and plays a crucial role in the linkage between integrin and the actin cytoskeleton, thereby regulating numerous aspects of cellular signaling in association with cell adhesion (37, 38). One of its roles in the complex appeared to be to distribute PINCH to focal adhesions, as reported previously (21). Despite this leading role, however, ILK required the presence of the C-terminal end of PINCH besides its own binding domain for regulation of the shuttling of the Hic-5 and PINCH complex study, we also found that ILK played an essential role in the regulation of focal adhesions and, thus likely plays a supportive role, possibly through the promotion and/or stabilization of the PINCH-ILK interaction. In this study, we further comprehensively studied the role of the Hic-5-directed organization of the Hic-5-organized nuclear-cytoplasmic shuttling complex and its primary role in the distribution of PINCH to focal adhesions, ILK probably facilitates the formation of a complex at focal adhesions and, hence, facilitates the Hic-5-directed organization of the shuttling complex would potentially contribute to an understanding of the regulation of cell growth/survival by cell adhesion.

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