J. Biochem. 132, 279–289 (2002)

Hic-5 Interacts with GIT1 with a Different Binding Mode from Paxillin[1]

Naoyuki Nishiya, Takeshi Shirai, Wataru Suzuki, and Kiyoshi Nose[2]

Department of Microbiology, Showa University School of Pharmaceutical Sciences, Hatanodai 1-5-8, Shinagawa-ku, Tokyo 142-8555

Received April 30, 2002; accepted June 4, 2002

Hic-5, a member of the paxillin family of adaptor molecules, is localized at focal adhesion and implicated in integrin-mediated signaling. Hic-5 and paxillin exhibit structural homology and share interacting factors, however, diverse functions are suggested for them. In this study, we carried out yeast two-hybrid screening to identify Hic-5 interacting factors using its LD3-4 region, which includes the Hic-5-specific amino acid sequence, as a bait. Through the screening, we identified GIT1, an Arf GTPase-activating protein, as a Hic-5 binding protein. The interaction of these two proteins was mediated by the LD3 motif of Hic-5 and the C-terminal region, which includes a paxillin-binding subdomain, of GIT1. Although GIT1 is known as a paxillin-binding protein, we only observed weak association of paxillin with GIT1 in the overexpression system. In contrast, Hic-5 firmly bound to GIT1 under the same conditions. In addition, the paxillin/GIT1 complex contained PIX, a guanine nucleotide exchange factor, whereas the Hic-5/GIT1 complex contained a smaller amount of PIX. These results suggested that paxillin and Hic-5 associate with GIT1 with different binding modes, and that the Hic-5 complex possesses static features compared with the paxillin complex, which contains both positive and negative regulators of GTPases involved in actin dynamics. Moreover, Hic-5–mediated inhibition of cell spreading was restored by co-expression of the C-terminal fragment of GIT1, which perturbs the interaction of Hic-5 with endogenous GIT1. Thus, it was demonstrated that Hic-5 and GIT1 interact functionally in addition to showing a physical association.

Key words: forcal adhesion, GIT1, Hic-5, paxillin.

Cell adhesion to the extracellular matrix (ECM) regulates multiple physiological events, such as cell migration, growth, and survival (*1, 2*). Cells respond to the ECM through transmembrane receptors, integrins, which activate intracellular signaling pathways that regulate the actin cytoskeleton and gene expression. Binding of integrins to the ECM induces clustering of these receptors, and recruitment of structural and signaling molecules to the specialized site on the inner cytoplasmic membrane to form focal complexes. These complexes provide a link between ECM-attachment and the cytoskeleton, and regulate intracellular signaling pathways, thereby coordinating cell-ECM attachment with cell architecture, motility, and gene expression (*1, 2*).

Paxillin is a multidomain adapter molecule that has been implicated in the regulation of integrin and growth factor signaling (*3*). Paxillin is tyrosine phosphorylated following integrin stimulation by focal adhesion kinase (FAK) and/or associated kinases (*4*). This phosphorylation creates docking sites for the SH2 domain of Crk (*4, 5*), and links integrin stimulation to downstream signaling pathways through GEFs, such as C3G (*6*) and DOCK180 (*7*). The structure of paxillin can be divided into two regions, the N-terminal region including five LD motifs (*8, 9*) and the C-terminal region comprising four LIM domains (*10*). Some of these structures have been recognized as binding sites for signaling molecules and structural proteins. In particular, the LD4 motif has been reported to bind to several molecules, such as FAK, vinculin, and paxillin kinase linker (PKL) (*8, 11*). PKL, which is a member of the GIT1 family of Arf-GAPs, links paxillin to a complex including PIX, PAK, and Nck (*11*). Moreover, the LD4 motif of paxillin regulates Rac activity, cell spreading, and motility through the interaction with PKL (*12*). Therefore, it has been proposed that the association of paxillin with PKL/PIX/PAK is involved in actin cytoskeletal dynamics mediated by both Arf and Rho family GTPases.

Hic-5, a member of the paxillin family of adapters, was originally isolated as a transforming growth factor β1 (TGFβ1)– and hydrogenperoxide-inducible gene by differential hybridization (*13*). It has been shown that Hic-5 is involved in cellular senescence (*14*) and differentiation (*15*).

[1] This work was supported in part by Grants-in-Aid for Science Research, a Grant-in-Aid for Cancer Research, and the High-Technology Research Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

[2] To whom correspondence should be addressed. Tel: +81-3-3784-8208, Fax: +81-3-3784-6850, E-mail: knose@pharm.showa-u.ac.jp

Abbreviations: Arf, ADP-ribosylation factor; ECM, extracellular matrix; FAK, focal adhesion kinase; GAP, GTPase-activating protein; GST, glutathione S-transferase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PAK, p21-activated protein kinase; PBS, paxillin-binding subdomain; PKL, paxillin-kinase linker; PIX, PAK-interacting exchange factor; PTP, protein tyrosine phosphatase; SH2, Src homology domain 2.

Similar to paxillin, Hic-5 is composed of an N-terminal half including four LD motifs and a C-terminal half comprising four LIM domains (*16*), and binds to FAK (*8*), PTP-PEST (*17*), vinculin (*8*), and PKL (*11*), which are shared with paxillin. Despite their similarity, Hic5 has some features distinct from those of paxillin. Unlike paxillin, Hic-5 does not possess a target site for the SH2 domain of Crk. Recent studies revealed that Hic-5 competes with paxillin for FAK (*18, 19*) and perturbs signaling pathways mediated by paxillin phosphorylation (*18*). Therefore, we proposed that the counterbalance of paxillin- and Hic5-expression can control the efficiency of integrin-mediated signal transduction (*18*).

The Rho family of small GTPases, such as Cdc42, Rac, and Rho, plays a crucial role in the actin cytoskeletal dynamics at the edges of migrating cells (*20–22*). Cell attachment to the ECM via integrins leads to rearrangement of the actin cytoskeleton and cell spreading, which is controlled by Cdc42 and Rac (*23*). Activation of Cdc42 and Rac stimulates the formation of filopodia, and lamellipodia and membrane ruffling, respectively (*24*). Numerous effector molecules have been implicated in Rho family GTPase-mediated signaling (*25*). p21-activated kinase, PAK is a serine/threonine kinase that is activated on interaction with Cdc42 and Rac (*26*), and has been implicated in both Cdc42- and Rac-mediated organization of the actin cytoskeleton (*27–30*). Conversely, Rac activation can occur through p21-interacting exchange factor (PIX), a guanine nucleotide exchange factor (GEF) (*31, 32*), and PAK-binding to PIX plays an important role in coordinating the activation of Cdc42 and Rac in a phosphatidylinositol 3-kinase–dependent manner (*33*).

PIX has been reported to associate with G protein–coupled receptor kinase–interacting target (GIT1), a member of a GTPase-activating protein (GAP) family for ADP-ribosylation factor (Arf) (*34*). This family of Arf-GAP includes GIT1 (*34*), GIT2 (*35*), PKL (*11*), and APP1 (*36*). These proteins contain an Arf-GAP domain in their N-terminal regions, and bind to paxillin that is localized at focal contacts (*11, 34–36*). Therefore, GIT1 family proteins may serve as a link between the PAK/PIX complex and focal contacts. Arf6 is co-localized with Rac at the plasma membrane and on recycling endosomes, and its GAP activity is required for the Rac-mediated function and transport to the membrane (*37*). Thus, Arf6 has been suggested to function in concert with Rac (*38*).

In this study, we identified GIT1 as a Hic-5 interacting factor using a yeast two-hybrid system. We show that GIT1 binds to both Hic-5 and paxillin directly *in vitro*, however, these associations in cells appear to be distinctively regulated. Furthermore, we also demonstrate that perturbation of the interaction between Hic-5 and GIT1 interferes with Hic-5–mediated inhibition of cell spreading.

EXPERIMENTAL PROCEDURES

Antibodies—Anti–Hic-5, anti-paxillin, anti-βPIX, and anti-GIT1 mouse monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-hemagglutinin (HA) polyclonal antibodies (Y11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hemagglutinin (HA) monoclonal antibodies (12CA5) were purchased from Boehringer Mannheim (Indianapolis, IN). Anti-Flag (M2) monoclonal antibodies were purchased from SIGMA (St. Louis, MO). Horseradish peroxidase (HRP)–conjugated anti–mouse IgG antibodies were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell Culture and Transfection—NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum and 50 μg/ml kanamycin at 37°C under an atmosphere of 5% CO_2 in air. Cos7 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin at 37°C under an atmosphere of 5% CO_2 in air. C2C12 cells were cultured in DMEM supplemented with 15% heat-inactivated calf serum and 50 μg/ml kanamycin at 37°C under an atmosphere of 5% CO_2 in air. Cells were transfected with plasmid DNAs using LipofectAMINE PLUS reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer's protocol.

Plasmids—Expression vectors for HA-tagged paxillin (pCG-pax), HA-tagged wild-type human Hic-5 (pCG-h*hic-5*), and HA-tagged Hic-5 mutants, HA-ΔLD3 (pCG-ΔLD3) and HA-ΔLD3-4 (pCG-ΔLD3-4), were described previously (*18*). The expression vector for C-terminal Flag-tagged GIT1 (pBKΔ-GIT1/Flag) (*34*) was from Dr. Richard Premont, Duke University Medical Center.

To construct an expression vector for a HA-tagged human Hic-5 LD4-deletion mutant that lacks amino acid residues 201 to 222 (pCG-ΔLD4), cDNAs encoding the N-terminal fragment and the C-terminal fragment were amplified by independent PCR. The cDNA encoding the N-terminal fragment, which corresponded to amino acid residues 1–200, was amplified using a 5′-primer incorporating a *Bam*HI site and a 3′-primer incorporating an *Eco*RI site. The cDNA encoding the C-terminal fragment, which corresponded to amino acid residues 223–461, was amplified using 5′-primers incorporating *Eco*RI sites and a 3′-primer incorporating a *Hin*dIII site. The PCR products encoding the N-terminal fragment and the C-terminal fragment were digested with *Eco*RI and ligated. The ligated fragments were digested with *Bam*HI and *Hin*dIII, and then incorporated into the expression vector driven by the CMV-promoter pCG-N-BL (*39*). For the construction of expression vectors for N-terminal Flag-tagged GIT1 and its deletion mutants (pFlag-GIT1, pFlag-GIT1 385–770, and pFlag-GIT1 637–770), selected regions of pBKΔ-GIT1/Flag were amplified by PCR using 5′-primers containing an *Eco*RI site and a 3′-primer incorporating an EcoRV site. The amplified fragments were digested with *Eco*RI and *Eco*RV, and then ligated into the corresponding sites on pFlag-CMV2 (SIGMA).

To construct a prokaryotic expression vector encoding a GST-fusion GIT1 C-terminal fragment that contains amino acid residues 628–671 (GST-GIT1 628–671), the corresponding region of pBKΔ-GIT1/Flag was amplified by PCR using 5′-primers containing an *Eco*RI site and a 3′-primer incorporating an *Eco*RV site. The amplified fragment was digested with *Eco*RI and *Eco*RV, and then ligated into the *Eco*RI/*Sma*I region on pGEX-5X (Amersham Pharmacia Biotech, Buckinghamshire, UK).

The identities of all inserts amplified by PCR were confirmed by sequencing.

Two-Hybrid Screening—A cDNA fragment that encodes from the LD3 motif to the LD4 motif of mouse Hic-5 (amino acid residues 113–190) was subcloned into pGilda (CLON-

TECH, Palo Alto, CA) and used as a bait. Approximately 1.4×10^7 cDNA clones derived from NIH3T3 cells were screened by means of a MATCHMAKER LexA two-hybrid system (CLONTECH), and cDNA clones that encode Hic-5–binding proteins were selected by growth on Lucien-deficient plates and by means of β-galactosidase activity. Positive clones were tested as to their two-plasmid dependency and sequenced using a Cy5 TM AutoRead™ sequencing kit (Amersham Pharmacia Biotech.).

Immunoprecipitation and Immunoblotting—Cells were washed with PBS and lysed in Triton lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM NaF, and protease inhibitor mixture, Wako, Tokyo), and then the insoluble material was removed by centrifugation. Cell lysates were pre-cleaved with normal rabbit or mouse IgGs (DAKO, Copenhagen, Denmark) immobilized on protein G-Sepharose (Amersham Pharmacia Biotech). Pre-cleared lysates were incubated with anti-HA (Y11), anti-Flag (M2), anti–Hic-5, or anti-paxillin antibodies immobilized on protein G-Sepharose at 4°C for 60 min. The immunocomplexes were then washed 4 times in Triton lysis buffer and boiled in SDS-PAGE loading dye.

For immunoblotting, proteins were resolved on SDS-PAGE and then electrophoretically transferred to PVDF membranes. The membranes were washed with phosphate-buffered saline (PBS), blocked with blocking buffer (PBS containing 0.1% Tween and 1% BSA), and then incubated with anti-HA (12CA5), anti-Flag (M2), anti-paxillin, anti–Hic-5, anti-GIT1, or anti-βPIX antibodies for 1 h at room temperature. The bound antibodies were detected with horseradish peroxidase–conjugated goat anti–mouse IgG antibodies (Amersham Pharmacia Biotech), and visualized with an enhanced chemiluminescence detection system (Renaissance TM; New England Nuclear Life Science Products, Boston, MA).

Immunodepletion—C2C12 cells were lysed in Triton lysis buffer, and insoluble material was removed by centrifugation. Cell lysates were pre-cleaved with normal mouse IgG. Endogenou Hic-5 was depleted by immunoprecipitations three times with anti–Hic-5 antibodies immobilized on protein G-Sepharose at 4°C for 1 h. Again, the residual supernatant (Hic-5–depleted lysate) was subjected to immunoprecipitation with anti-paxillin antibodies immobilized on protein G–Sepharose at 4°C for 1 h. The immunocomplexes precipitated with these antibodies were separated by SDS-PAGE, and detected by immunoblotting with anti-GIT, anti-βPIX, or anti-paxillin antibodies.

In vitro Binding Assay—The GST-fusion proteins were expressed by incubation of *E. coli* strain DH5α harboring the expression vectors with 0.5 mM isopropyl-D-thiogalactopyranoside for 3 h at 30°C. The bacteria were collected by centrifugation, and lysed in PBS containing 100 μg/ml lysozyme, 1% Triton X-100, 1 mM PMSF, and protease inhibitor mixture (Wako, Tokyo). The fusion proteins were purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech).

^{35}S-labeled Hic-5 and paxillin were synthesized *in vitro* using a TNT® T7 coupled reticulocyte lysate system (Promega, Madison, WI) in the presence of [^{35}S]methionine. Two expression vectors that bore the T7 promoter sequence, pcDNA3.1A-h*hic-5* (*18*) and pRC/CMV/paxillin (*40*), were used as templates. The *In vitro* translated products were then incubated with immobilized GST-fusion proteins in the binding buffer (20 mM Tris-HCl pH 7.4, 1% Triton X-100, 1% BSA, 1 mM PMSF, and protease inhibitor mixture, Wako, Tokyo) for 1 h at 4°C. The beads were washed four times with the binding buffer. The bound fractions were eluted with the elution buffer (50 mM Tris-HCl pH 8.0, 0.2 M KCl, and 20 mM glutathione), and then subjected to SDS-PAGE. After drying, the gels were exposed to X-ray films.

Cell Spreading Assay—The cell spreading assay was performed as described previously (*18*). Briefly, NIH3T3 cells were transiently transfected with GFP expression vector pEGFP-N3 together with expression vectors, as described in the figure legends. Cells resuspended in serum-free DMEM containing 1% BSA were replated on coverslips coated with 5 μg/ cm^2 fibronectin (Upstate Biotechnology, NY) and then placed on ice for 5 min. Subsequently, the cells were allowed to spread for 30 min at 37°C. Cells attached to coverslips were fixed with 3.7% formaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, and then stained with TRITC-conjugated phalloidin (Sigma). After washing with PBS containing 0.1% Tween 20, the cells were mounted and visualized under a low magnification fluorescence microscope. GFP-positive cells were counted as transfected cells, and the percentage of spread cells was determined. Nonspread cells were defined as round phase-bright cells, whereas spread cells were defined as those that lacked a rounded shape, that were not phase-bright, and that had extended membrane protrusions. In each experiment, more than 150 cells were counted. At least 3 independent experiments were performed for each combination of experiments.

RESULTS

Isolation of cDNA Clones Encoding Hic-5–Binding Proteins—To investigate the molecular basis of Hic-5–mediated cellular function, we attempted to isolate Hic-5 associated proteins by yeast two-hybrid screening (*41*). Several signaling molecules, such as vinculin (*8*), FAK (*8*), Pyk2/Cakβ/RAFTK (*42*), and p95PKL (*11*), are known to bind to the LD4 motif of paxillin, which corresponds to the LD3 motif of Hic-5. Therefore, the LD3 motif of Hic-5 was expected to be a binding site for other molecules, which had not been reported previously. Even though Hic-5 and paxillin are structurally homologous and share some binding factors, several findings have suggested that they have distinct functions (*18, 43*). For example, the spacer sequence between the LD3 and LD4 motifs, which includes a Hic-5–specific proline-rich sequence, was a candidate bait for screening the Hic-5–specific binding partner (Fig. 1, A and B).

We screened 1.4×10^7 clones of a NIH3T3-derived cDNA library with the region from the LD3 to the LD4 motif of Hic-5 as a bait. As a consequence of the screening, two independent positive clones were isolated, and their sequences showed identity to parts of previously cloned cDNA, G-protein-coupled receptor kinase-interacting target 1 (GIT1) (*34*). GIT1 is a member of the Arf-GAP family and closely related to GIT2/PKL (*11, 35*). The structure of GIT1 is characterized by the N-terminal Arf-GAP domain, four ankyrin repeats, and the yeast Spa2 homology domain 1 (SHD-1), as described previously (*34, 44*) (Fig. 1C). GIT1

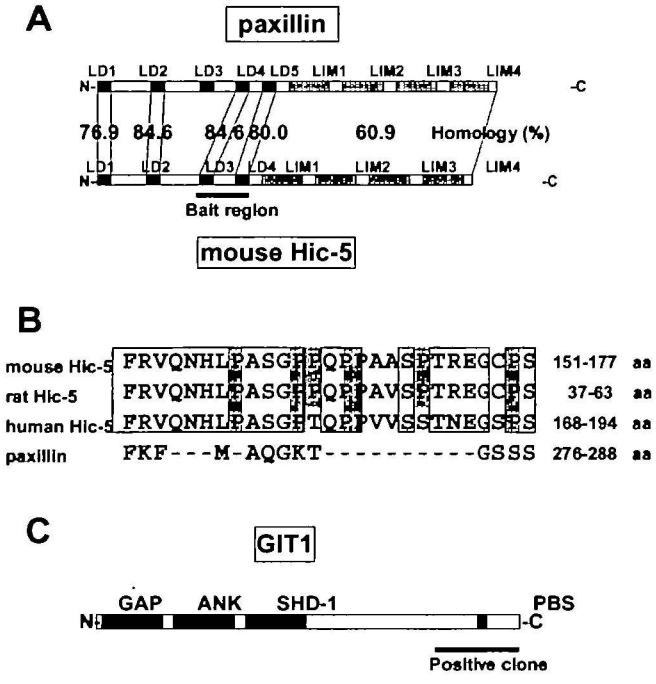

Fig. 1. **Structures of paxillin, Hic-5 and GIT1.** A: Comparison of the domain structures of paxillin and Hic-5. LD motifs are indicated as black boxes and LIM domains as shaded boxes. The percent identities between different regions of paxillin and the corresponding regions of mouse Hic-5 are indicated. The region used as a bait for two-hybrid screening is underlined. B: Sequence alignment of Hic-5–specific proline-rich regions. Conserved amino acid residues among mice, rats, and humans are boxed. Proline residues are highlighted in shaded boxes. The corresponding region of paxillin is also shown. C. Schematic representation of GIT1. The Arf-GAP domain (GAP), a region containing four ankyrin repeats (ANK), yeast Spa2-homology domain 1 (SHD1), and the paxillin-binding subdomain (PBS) are indicated. The clone isolated through two-hybrid screening encoded the underlined region.

was reported to interact with paxillin (*35, 44*). However, it had not been clarified whether Hic-5 can bind to GIT-1, or whether there are any differences in the binding mode between Hic-5 and paxillin. Therefore, we performed to further analysis on the binding of Hic5 to GIT1.

Hic-5 and GIT1 Associate in Mammalian Cells—To confirm the association of Hic-5 with GIT1 in mammalian cells, we introduced expression vectors that encode Flag-tagged GIT1, HA-tagged Hic-5 and HA-tagged paxillin into Cos7 cells, and examined their association by immunoprecipitation and immunoblotting. HA-tagged Hic-5 or HA-tagged paxillin was immunoprecipitated with anti-HA antibodies. In the presence of HA-tagged Hic-5, Flag-tagged GIT1 was co-precipitated (Fig. 2A). However, the co-precipitation of GIT1 with HA-tagged paxillin could hardly be detected in the present system. For immunoprecipitation with anti-Flag antibodies, HA-tagged Hic-5 was co-precipitated in a Flag-tagged GIT1-dependent manner (Fig. 2B). Co-precipitation of HA-tagged paxillin could scarcely be detected, as shown in Fig. 2A. However, long exposure on anti-HA antibody blotting revealed co-precipitation of HA-tagged paxillin with Flag-tagged GIT1 (Fig. 2B, second panel). These results showed that Hic-5 associates with GIT1 in mammalian cells, and suggested that Hic-5 can

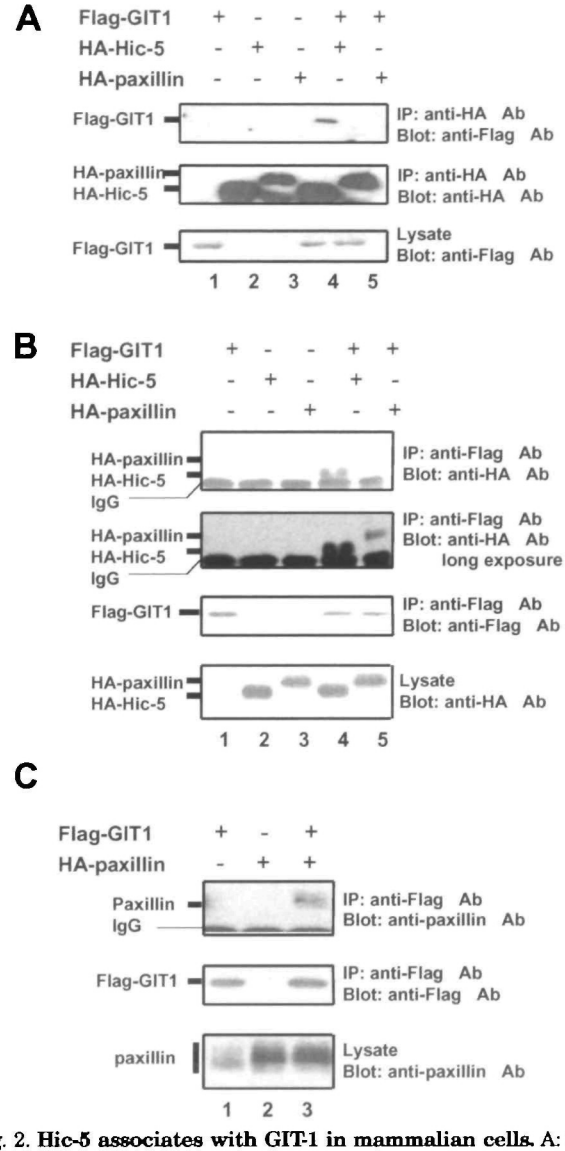

Fig. 2. **Hic-5 associates with GIT-1 in mammalian cells.** A: Cos7 cells were transfected with mammalian expression vectors engineered to express HA-tagged Hic-5 (pCG-h*hic-5*, lanes 2 and 4), HA-tagged paxillin (pCG-*pax*, lanes 3 and 5), or Flag-tagged GIT1 (pBKΔ-GIT1/Flag, lanes 1, 4, and 5). Twenty-four hours after transfection, cells were lysed and immunoprecipitated (IP) with anti-HA antibodies. Immunocomplexes were subjected to SDS-PAGE and immunoblotting (blot) with anti-Flag (upper panel) or anti-HA (middle panel) antibodies. In the lower panel, cell lysates were subjected to SDS-PAGE and immunoblotting with anti-Flag antibodies. B: Cos7 cells were transfected with the HA-tagged Hic-5 (pCG-h*hic-5*, lanes 2 and 4), HA-tagged paxillin (pCG-*pax*, lanes 3 and 5), or Flag-tagged GIT1 (pBKΔ-GIT1/Flag, lanes 1, 4, and 5) expression vector. Cell lysates were prepared and subjected to immunoprecipitation with anti-Flag antibodies and immunoblotting with anti-HA (top and second panels) or anti–Flag (third panel) antibodies. In the second panel, the X-ray film was exposed 5 times longer than in the upper panel. In the bottom panel, cell lysates were subjected to SDS-PAGE and immunoblotting with anti-HA antibodies. C. Cos7 cells were transfected with the Flag-tagged GIT1 (pBKΔ-GIT1/Flag, lanes 1 and 3) or HA-tagged paxillin (pCG-*pax*, lanes 2 and 3) expression vector. Cell lysates were prepared and subjected to immunoprecipitation with anti-Flag antibodies and immunoblotting with anti-paxillin (upper panel) or anti–Flag (middle panel) antibodies. In the lower panel, cell lysates were subjected to SDS-PAGE and immunoblotting with anti-paxillin antibodies.

form a complex with GIT1 more stably and/or tightly than paxillin. However, Cos7 cells express considerable levels of endogenous paxillin, but Hic-5 was hardly detectable. To exclude competitive effects for the association with GIT1 between endogenous and exogenous paxillin, co-precipitation of endogenous paxillin with Flag-tagged GIT1 was also examined. We could also scarcely detect endogenous paxillin in the Flag-tagged GIT1 immunocomplex, while exogenous paxillin was detected (Fig. 2C). This suggests that endogenous paxillin cannot compete with overexpressed paxillin, and that paxillin is a faint binding partner for Flag-GIT1.

Domains for Interaction between Hic-5 and GIT1—The region used as a bait contained the LD3 and LD4 motifs of Hic-5. Since it was reported that a single LD motif acts as a binding site for interacting factors (*8, 11*), we tried to map the LD motif required for GIT1-binding. HA-tagged Hic-5 mutants with the LD3 and/or LD4 motifs deleted (HA-ΔLD3, HA-ΔLD4, and HA-ΔLD3-4) were co-expressed with Flag-tagged GIT1 in Cos7 cells. HA-tagged Hic-5 mutants were immunoprecipitated with anti-HA antibodies, and co-precipitated Flag-tagged GIT1 was analyzed by immunoblotting with anti-Flag antibodies. HA-ΔLD4 formed a complex with Flag-GIT1 with comparable strength to the wild-type Hic-5 (Fig. 3B, compare lanes 2 and 4). In contrast, HA-ΔLD3 and HA-ΔLD3-4 hardly associated with Flag-GIT1 (Fig. 3B, lanes 3 and 5). These results suggest that the LD3 motif of Hic-5 is critical for physical interaction with GIT1. However, a small quantity of co-precipitated GIT1 was observed in the HA-ΔLD3 and HA-ΔLD3-4 immunocomplexes on long exposure on anti-Flag antibody blotting. This may reflect the presence of bridging proteins that form a complex including Hic-5 LD mutants and GIT1. The LD3 motif of Hic-5 and the LD4 motif of paxillin exhibit particularly high homology in amino acid sequence (91.7% identical), nevertheless their efficiencies to form complexes with GIT1 are quite different. This difference may be due to the region surrounding the LD3 motif of Hic-5.

One of the clones, which was isolated through the two-hybrid screening, encoded amino acid residues 535–770, which include the region reported to be a paxillin-binding subdomain (amino acid residues 682–701) (*11, 45*). It was

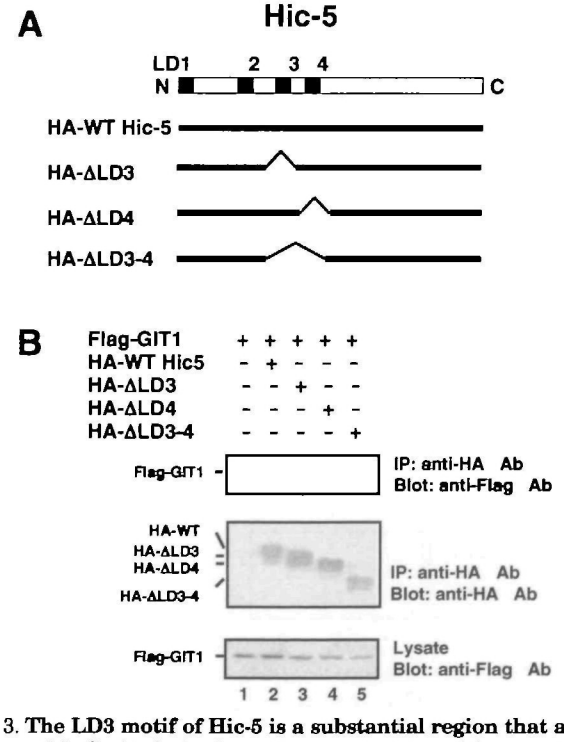

Fig. 3. **The LD3 motif of Hic-5 is a substantial region that associates with GIT-1.** A: A schematic representation of the mutations in the LD motifs of Hic-5. Four LD motifs are indicated. B: Cos7 cells were transfected with the Flag-tagged GIT1 expression vector (pFlag-CMV2/GIT1) together with the empty vector (lanes 1 and 6) or an expression vector encoding HA-tagged wild-type Hic-5 (pCG-h*hic-5*, lanes 2 and 7), HA-tagged Hic-5 with the LD3 motif deleted (pCG-ΔLD3h*hic-5*, lanes 3 and 8), HA-tagged Hic-5 with the LD4 motif deleted (pCG-ΔLD4h*hic-5*, lanes 4 and 9), or HA-tagged Hic-5 with the LD3-4 motif deleted (pCG-ΔLD3-4h*hic-5*, lanes 5 and 10). Cells were lysed and subjected to immunoprecipitation with anti-HA antibodies and immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibodies.

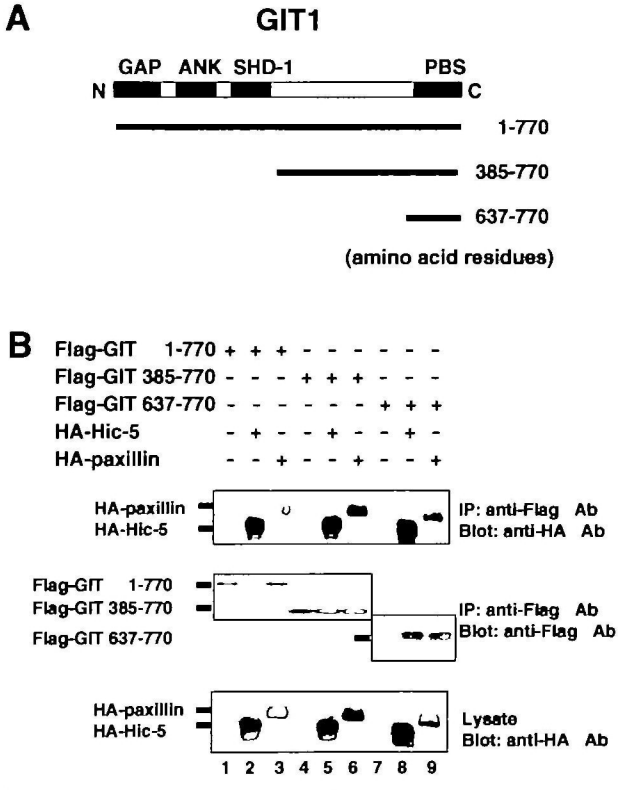

Fig. 4. **The C-terminal region of GIT1 is sufficient for association with Hic-5.** A: A schematic representation of the GIT1 deletion mutant. The Arf GAP domain (GAP), four ankyrin repeats (ANK), the yeast Spa2 homology domain 1 (SHD), and the paxillin-binding domain (PBD) are indicated. B: Cos7 cells were transfected with expression vector pFlag-GIT1 (Flag-tagged full length GIT1, lanes 1–3), pFlag-GIT 385–770 (Flag-tagged GIT1 with 1-384 amino acid residues deleted, lanes 4–6), or pFlag-GIT1 637–770 (Flag-tagged GIT1 1–636 amino acid residues deleted, lanes 7–9) together with the empty vector (lanes 1, 4, and 7) or expression vectors pCG-h*hic-5* (HA-tagged Hic-5, lanes 2, 5, and 8) or pCG-*pax* (HA-tagged paxillin, lanes 3, 6, and 9). Cells were lysed and subjected to immunoprecipitation with anti-Flag antibodies and immunoblotting with anti-HA (upper panel) or anti-Flag (middle panel) antibodies. In the lower panel, cell lysates were subjected to SDS-PAGE and immunoblotting with anti-HA antibodies.

recently reported that the C-terminal fragment of GIT1 was necessary and sufficient for association with paxillin (*44*). To determine whether this is applicable to the association of Hic-5 with GIT1, we localized the Hic-5-binding region on GIT1 by co-transfection of Flag-tagged GIT1 fragments with HA-tagged Hic-5 (Fig. 4). Similar to paxillin, Hic-5 associated with the C-terminal 134 amino acid residues (GIT 637–770) of GIT1 as efficiently as the larger fragment, GIT 385–770 (Fig. 4B). Although the full-length GIT1 was a poor paxillin-interactor, as reported previously (*44*), Hic-5 associated with the full-length GIT1 with comparable strength to smaller fragments (Fig. 4B, compare lanes 2 and 3). Since coupling of GIT1 to PIX, PAK-interacting guanine exchange factor, which is involved in regulation of the cytoskeletal organization (*32*), promotes the association between paxillin and the full-length GIT1 (*44*), it has been suggested that the paxillin-binding domain of GIT1 is masked in the absence of PIX, and opened by PIX (*44*). Therefore, these observations suggested the association between Hic-5 and the C-terminal region of GIT1 is different in part from that of paxillin and GIT1.

Direct Binding of GIT1 to Hic-5—To determine whether Hic-5 binds directly to GIT1, ^{35}S-labeled Hic-5 was synthesized *in vitro* using a cell-free transcription/translation system in the presence of [^{35}S]methionine. ^{35}S-labeled Hic-5 was incubated with the GST-fusion protein of GIT1 C-terminal fragment 637–770, which was immobilized on glutathione-Sepharose beads. The bound proteins were eluted and detected by SDS-PAGE, followed by fluorography. ^{35}S-labeled Hic-5 was recovered as a bead-bound fraction in the presence of the C-terminal fragment of GIT1 (GIT 637–770) (Fig. 5). This result demonstrated that Hic-5 binds directly to the GIT 637–770 fragment. Paxillin also bound directly to GIT1, with similar affinity to in the case of the Hic-5/GIT1 interaction. Thus, the intramolecular interaction in GIT1 and its regulation by PIX appear to be more important as to the efficiency to form the GIT1/paxillin complex than the affinity of paxillin to the C-terminal region of GIT1.

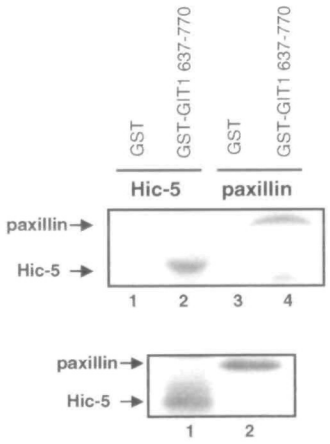

Fig. 5. **Hic-5 binds directly to the C-terminal fragment 637-770 of GIT1.** Hic-5 and paxillin protein were *in vitro* translated in the presence of [^{35}S]methionine, and then incubated with glutathione-Sepharose beads coupled with GST (upper panel, lanes 1 and 3) or the GST-fusion GIT1 637–770 fragment (upper panel, lanes 2 and 4) for 60 min. After washing, bound proteins were eluted with the elution buffer containing 20 mM glutathione, and then subjected to SDS-PAGE and autoradiography (12 h exposure). In the lower panel, 10% of the amount of each *in vitro* translated product used in the binding assay was subjected to SDS-PAGE and autoradiography (12 h exposure).

Association of Endogenous GIT1 with Hic-5 and Paxillin—It has been suggested that the binding of paxillin to GIT1 depends on PIX (*44*), nevertheless Hic-5 can efficiently associate with GIT1 in the absence of PIX overexpression (Figs. 2 and 4). Therefore, it was suggested that the Hic-5/GIT1 complex contains lower levels of PIX than the paxillin/GIT1 complex, and that this could be a possible mechanism of the functional difference between Hic-5 and paxillin. Since it has been suggested that stoichiometric balances among these proteins are critical, endogenous protein complexes were compared by immunoprecipitation with anti–Hic-5 and anti-paxillin antibodies. As described previously, the expression levels of Hic-5 are decreased in immortalized cell lines (*14, 43*), but mouse myoblastic cells, C2C12, express significant amounts of Hic-5. Anti-paxillin antibodies exhibit cross-reactivity to Hic-5, but anti-Hic5 antibodies are relatively specific for Hic-5. Thus, to observe Hic-5– and paxillin-complexes separately, endogenous Hic-5 was depleted by serial immunoprecipitations with anti-Hic-5 antibodies (Fig. 6A). Co-precipitated proteins were

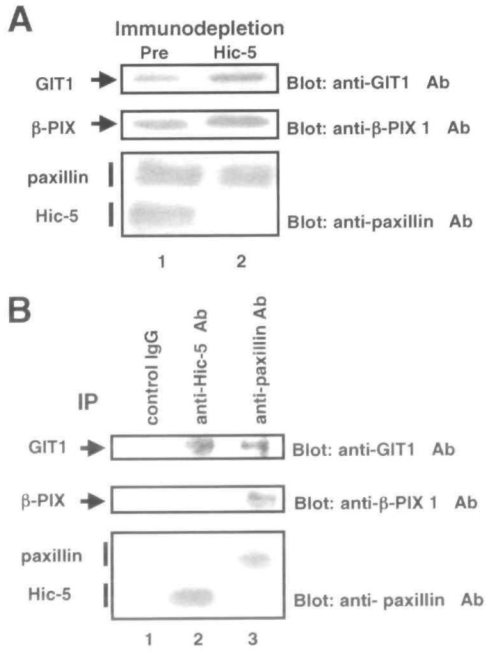

Fig. 6. **Hic-5/GIT1 complex contains a smaller amount of PIX than the paxillin/GIT1 complex.** A: Endogenous Hic-5 was depleted by multiple immunoprecipitation with anti–Hic-5 antibodies (lane 2) or normal mouse IgG (lane 1), as a control, from a C2C12 cell lysate. Mock- and Hi-5–depleted lysates were subjected to SDS-PAGE and immunoblotting with anti-GIT (upper panel), anti-βPIX (middle panel), or anti-paxillin (lower panel) antibodies. B. A mock-depleted lysate was subjected to serial immunoprecipitation with ant–Hic-5 antibodies. The residual supernatant (Hic-5–depleted lysate) was subjected to further immunoprecipitation with anti-paxillin antibodies. Co-precipitated proteins with normal mouse IgG (lane 1), anti–Hic-5 (lane 2), or anti-paxillin (lane 3) antibodies were separated by SDS-PAGE, and detected by immunoblotting with anti-GIT (upper panel), anti-βPIX (middle panel), or anti-paxillin (lower panel) antibodies.

recognized as components of the Hic-5-complex (Fig. 6B, lane 2), and Hic-5-depleted supernatant was then immunoprecipitated with anti-paxillin antibodies (Fig. 6B, lane 3). Compared with the paxillin-complex, the Hic-5-complex contained larger amounts of endogenous GIT1 (Fig. 6B, the upper panel). However, complex formation between endogenous GIT1 and paxillin was observed and appeared to be more efficient than that between exogenous GIT1 and paxillin (compare Figs. 2B and 6B). The stoichiometric balances between GIT1 and PIX may explain these observations. Moreover, the relative amount of PIX, which is included in the paxillin-complex, was greater than that in the Hic-5-complex (Fig. 6B, middle panel). These results suggested that Hic-5 can interact with GIT-1 unconnectedly the presence of PIX on GIT1, and that the Hic-5- and paxillin-complexes contain different components. Since PIX and its interacting kinase, PAK, activate Rac1 that regulates cytoskeletal organization (*32, 33*), the above results suggested that the Hic-5/GIT1 and paxillin/GIT1 complexes may have different functions in regulation of the actin cytoskeleton.

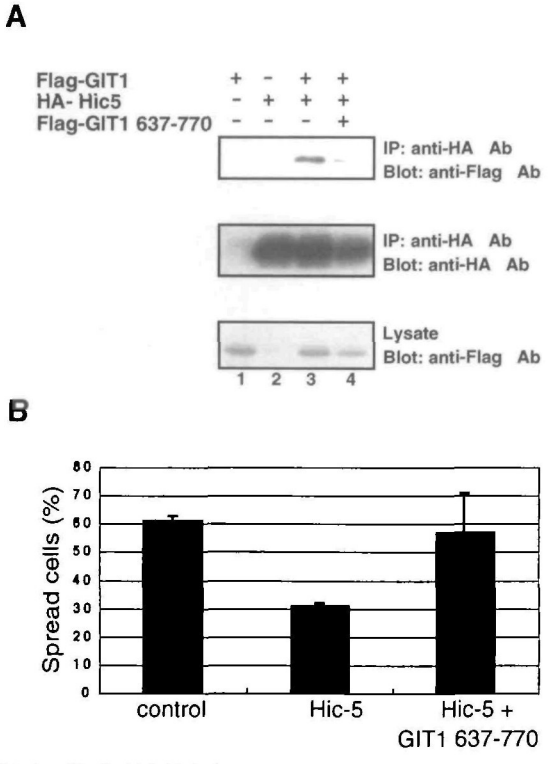

Fig. 7. **The GIT1 637–770 fragment perturbs the interaction of Hic-5 with endogenous GIT1 and the function of Hic-5.** A: Cos7 cells were transfected with the empty vector (lane 1) or the expression vector encoding HA-Hic-5 (lanes 2, 3, and 4) together with the empty vector (lanes 1, 2, and 3) or the expression vector encoding Flag-GIT 637–770 (lane 4). Cells were lysed and subjected to immunoprecipitation with anti-HA antibodies and immunoblotting with anti-Flag (upper panel) or anti-HA (middle panel) antibodies. In the lower panel, cell lysates were subjected to SDS-PAGE and immunoblotting with anti-Flag antibodies. B: NIH3T3 cells were transfected with the expression vector encoding EGFP together with the empty vector or the expression vector encoding Hic-5 with or without the Flag-GIT1 637–770 expression vector. After 24 h, cells were allowed to spread on fibronectin-coated coverslips for 30 min, fixed with 3.7% formalin, and then stained with TRITC-conjugated phalloidin. The percentages of spread cells on GFP-positive cells were determined. Each bar represents the mean of three independent experiments ± SD.

Functional Interaction between Hic-5 and GIT1—The GIT 637–770 fragment can bind directly to Hic-5 (Fig. 5), but does not contain binding sites for other interacting factors such as PIX, PAK, and FAK. Therefore, it is suggested that this GIT1 fragment could be used as a tool for perturbing the docking of GIT1 to Hic-5 in living cells. To confirm this possibility, NIH3T3 cells were transiently transfected with expression vectors encoding HA-tagged Hic-5 and Flag-tagged full length GIT1 together with or without Flag-tagged GIT 637–770. In the absence of Flag-GIT 637–770, the association of Flag-GIT1 with HA-Hic-5 was observed (Fig. 7A, lane 3). However, co-expression of Flag-GIT 637–770 prevented Hic-5 from forming the Hic-5/GIT1 complex (Fig. 7A, lane 4). This result showed that the GIT 637–770 fragment can be used to prevent endogenous GIT1 from interacting with Hic-5 in living cells.

We previously reported that the overexpression of Hic-5 inhibited integrin-mediated cell spreading on fibronectin in NIH3T3 cells (*18*). To examine the role of the physical interaction between GIT1 and Hic-5 in the Hic-5–mediated reactions, we examined the effect of the GIT 637–770 fragment on Hic-5–mediated inhibition of cell spreading. We introduced expression vectors encoding EGFP and HA-tagged Hic-5 with or without the expression vector for Flag-GIT 637–770 into NIH3T3 cells. Cells were collected by trypsinization, replated onto coverslips coated with fibronectin, and then fixed after 30 min incubation. EGFP-positive cells were counted as transfected cells, and the percentages of spread cells were estimated. As reported previously (*18*), the forced expression of Hic-5 caused about 50% inhibition of cell spreading compared with in the case of mock transfection cells (Fig. 7, compare columns 1 and 2). However, Hic-5–mediated inhibition of cell spreading was restored by co-expression of GIT 637–770 (Fig. 7, column 3). These results suggested that Hic-5 interacts functionally with GIT1 in living cells.

DISCUSSION

Hic-5, a member of the paxillin family of adapter proteins, has been shown to be involved in integrin-mediated signaling (*18*) as well as in the cellular senescence process or differentiation (*14, 15*). In the present study, we isolated GIT1 as a Hic-5–interacting protein using a two-hybrid system, and demonstrated that Hic-5 interacts physically and functionally with GIT1, and that Hic-5 and GIT1 form a complex with different binding modes from in the case of the paxillin/GIT1 complex.

GIT1 was originally identified as an interacting factor for the G protein-coupled receptor kinase, and is involved in signaling and internalization of the β_2-adrenergic receptor (β_2AR) (*34*). This protein is a member of an Arf-GAP family, which includes closely related members PKL/GIT2 (*11, 35*) and APP1 (*36*), and more distantly related members ASAP1 (*46*) and PAG3 (*47*). GIT1 possesses Arf-GAP activity and has been implicated in membrane trafficking events that are associated with the functions of Arf GTPases. In addition to trafficking of β_2AR, it was recently reported that GIT1 also regulates focal complex disassembly and promotes motility (*44*). GIT1 directly interacts with

paxillin and causes loss of paxillin from focal complexes, thereby contributing to focal contact disassembly and cell migration (*44*). In this sense, it is not surprising that GIT1 binds to and interacts functionally with Hic-5.

An LD motif is characterized by a conserved leucine-rich amino acid sequence with consensus sequence LDXLLXXL (*8*). It has been reported that individual LD motifs can function independently to support the binding of different proteins such as FAK, vinculin, PKL, and the E6 oncoprotein (*8, 11, 48, 49*). The LD4 motif of paxillin directly binds to PKL (*11*), and the association of Hic-5 with GIT1 appears to be mediated by its LD3 motif (Fig. 4), which corresponds to the LD4 motif of paxillin, which exhibit 91.7% identity in amino acid sequence to each other.

Paxillin associated with the C-terminal 134 residues of GIT (GIT 637–770) that includes a paxillin-binding subdomain (*11, 45, 50*), but full-length GIT1 exhibited only weak association with paxillin (Fig. 4). Since an association of GIT1 and PIX increases the association between paxillin and GIT1, it has been suggested that the paxillin-binding domain on GIT1 is masked in the absence of PIX and uncovered on the binding of PIX (*44*). In contrast, Hic-5 was associated with full-length GIT1 with a similar intensity to GIT 637–770 (Fig. 4). This difference in the GIT1-binding mode may be due to the different structures surrounding the GIT1-binding LD motifs (the LD3 motif of Hic-5 and the LD4 motif of paxillin). These LD motifs are highly conserved, as described above, however, their flanking regions have distinct amino acid sequences. In particular, the LD3 motif of Hic-5 is sandwiched between two proline-rich regions, which do not exist in the corresponding regions of paxillin. Since a proline residue tends to break the α-helix structure, it is suggested that the region neighboring the LD3 motif of Hic-5 can form an unusual structure, and thereby Hic-5 and paxillin show different GIT1-binding modes.

Deletion mutants of Hic-5 (ΔLD3 and ΔLD3-4), which lack the LD3 motif, hardly bound to GIT1, but very low levels of association between these mutants and GIT1 could be detected on long exposure (data not shown). This may reflect the existence of bridging proteins that link Hic-5 and GIT1. Since FAK can bind directly to GIT1 (*44*) and the LD2 motif of Hic-5 (*43*), it is suggested that FAK is a candidate for the bridging protein. Hic-5 mutants lacking the LD3 motif, one of FAK-binding regions on Hic-5, failed to inhibit cell spreading (*18*). Even if the LD2 motif of Hic-5 is another FAK-binding site, ΔLD1-2 Hic-5, which lacks the LD1 and LD2 motifs, could inhibit spreading as strongly as the wild-type Hic-5 (*18*). Since GIT1 interacts more specifically with the LD3 motif of Hic-5, Hic-5–mediated inhibition of cell spreading may be due to interaction with GIT1 rather than FAK.

Most adherent cells respond to extracellular matrices by adhering and spreading out to form new adhesion complexes and thereby acquired flattened morphology. This process is mediated by integrins and dynamic rearrangement of the actin cytoskeleton that is organized by intracellular signaling (*51, 52*). Cell spreading has been recognized as a convenient model of the initial state in cell migration (*53*). We previously reported that Hic-5 reduces cell spreading (*18*) and observed that Hic-5 opposes cell migration (unpublished data). Tyrosine phosphorylation of paxillin has been implicated in the regulation of cell migration (*54, 55*). Forced expression of Hic-5 removes FAK from paxillin and reduces the phosphorylation of tyrosine residues of paxillin that create target sites for Crk-SH2 (*18*). Furthermore, it has been suggested that Hic-5–mediated inhibition of cell spreading was FAK-dependent because the effect of Hic-5 was not observed in FAK–/– cells (*18*). Thus, we have recognized FAK as a critical factor for Hic-5–mediated functions. In addition, GIT1 promotes cell motility in concert with FAK and paxillin (*44*). It has been proposed that GIT1-induced cell migration is mediated by recruitment of GIT1 to focal contacts through paxillin, and activation of Rac1 by complex formation with PIX and FAK (*44*). Moreover, Hic-5–mediated inhibition of cell spreading appears to require the interaction with GIT1, as shown in Fig. 7. Therefore, it is suggested that the competition of the FAK/GIT1 complex between Hic-5 and paxillin is a possible mechanism for Hic-5–mediated inhibition of cell spreading (Fig. 8).

Rho family GTPases, such as RhoA, Rac1, and CdC42, regulate the actin cytoskeleton (*20–22*). Among these GTPases, Rac1 and Cdc42 are necessary for early spreading events characterized by a change in the cytoskeleton and extension of membrane protrusions (*23*). It has been reported that the interaction between PKL, a GIT-related Arf-GAP, and paxillin is critical for regulation of the Rac-

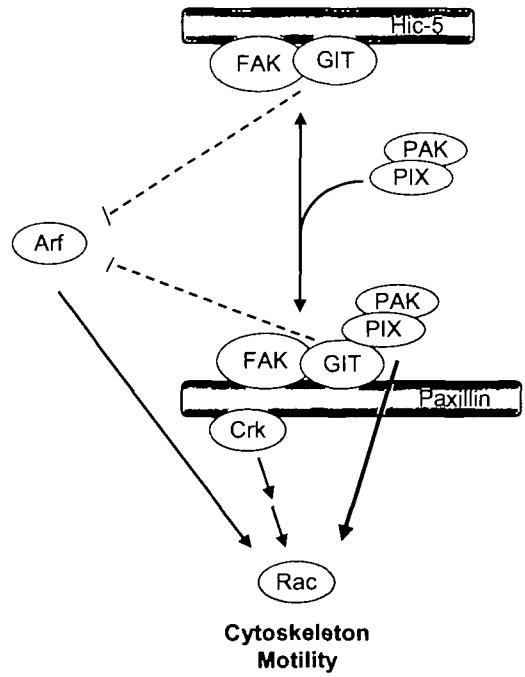

Fig. 8. **A model for the formation of different signaling complexes through Hic-5 and paxillin.** The paxillin/GIT1 complex contains PIX and PAK that activate Rac-mediated cytoskeletal rearrangement. Paxillin is phosphorylated by FAK and/or associated kinases, and then interacts with the SH2 domain of Crk, which transduces downstream signaling to Rac. In addition, Arf-6 regulates the translocation of Rac to the plasma membrane. GIT1 may downregulate Arf6 through its GAP activity. Therefore, the paxillin/GIT1 complex may show dynamic features through these positive and negative regulators of the cytoskeleton. On the other hand, Hic-5 does not possess a target site for the SH2 domain of Crk, and the Hic-5/GIT1 complex contains smaller amounts of PIX. Thus, the Hic-5 complex may show more static features compared with the paxillin complex.

dependent cell shape change and motility (*12*). GIT1 interacts with the PIX/PAK complex that activates Rac1 (*32*). PIX can activate Rac1 through the interaction with PAK and PI3-kinase (*33*). In addition, PAK can inhibit myosin light chain kinase (MLCK) (*56*) and subsequently reduce actin-myosin contractility. Thereby, the PIX/PAK complex facilitates cell motility. Paxillin requires PIX to open the masked paxillin-binding site of GIT1 (*44*). Thus, the GIT1/PIX/PAK complex may be formed on paxillin, and then promote focal contact turnover, cell spreading, and migration. However, Hic-5 appears to associate with GIT1 without or with only small amounts of PIX (Fig. 4). Therefore, it is suggested that Hic-5 and paxillin form different complexes consisting of different components. Indeed, co-precipitated PIX in the Hic-5 complex was less than that in the paxillin complex (Fig. 6B). This finding suggested that Hic-5 does not efficiently promote PIX/PAK-mediated processes, such as Rac activation, and this process may contribute to Hic-5–mediated inhibition of cell spreading (Fig. 8). On the other hand, it has been reported that Hic-5 interacts with PKL together with PIX and PAK (*11*). However, the efficiencies of complex formation with PKL/PIX/PAK have not been compared between paxillin and Hic-5. In addition, GIT1 possesses a Spa2 homology domain (SHD), which binds to FAK and is absent from PKL/GIT2, and is phosphorylated on co-transfection with FAK or Src (*44*). The SHD is located close to the PIX-binding site, nevertheless, GIT1 can bind to FAK and PIX simultaneously (*44*). It is important to compare Hic-5 with paxillin with regard to the intensity, specificity, effects of phosphorylation and PIX-dependency in binding to these Arf-GAPs. This may lead to new concepts as to the regulation system in actin cytoskeleton dynamics.

The precise spatial regulation of signaling molecules has been suggested to be critical for proper organization of the cytoskeleton. Rac-mediated regulation of actin cytoskeleton dynamics has been linked to its translocation to the membrane (*57, 58*). It has been proposed that Arf6 regulates the translocation of Rac to the membrane (*37, 38, 59*). Moreover, p95-APP1, an Arf-GAP closely related to GIT1 and PKL, induces actin-rich protrusions mediated by Rac and Arf6 (*36*). Since it has been suggested that GAP-activity opposes GTPase-mediated functions by facilitating GTP hydrolysis, antagonism toward Arf6 and Rac through the Arf-GAP activity of GIT1 is a possible mechanism of Hic-5–mediated reduction of cell spreading (Fig. 8).

In conclusion, our observations demonstrated that Hic-5 interacts physically and functionally with GIT1. Hic-5 shares structural features and interaction factors with paxillin. In this study, we added GIT1 to the list of proteins that bind to both Hic-5 and paxillin. However, the paxillin/GIT1 complex contains different components, which regulate cytoskeletal remodeling, from the Hic-5/GIT1 complex. Further studies on these complexes are expected to clarify the nature of the signaling complexes as functional units in integrin-mediated signaling.

We are grateful to Dr. Richard T. Premont (Duke University Medical Center, Durham, NC) for providing the GIT1 expression vector. Cos7 cells were provided by the RIKEN Cell Bank. We also thank Takahiro Ohki and Ikumi Kikuchi for the technical assistance.

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