Hic-5, a Paxillin Homologue, Binds to the Protein-tyrosine Phosphatase PEST (PTP-PEST) through Its LIM 3 Domain*

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The Hic-5 protein is encoded by a transforming growth factor-β1- and hydrogen peroxide-inducible gene, hic-5, and has striking similarity to paxillin, especially in their C-terminal LIM domains. Like paxillin, Hic-5 is localized in focal adhesion plaques in association with focal adhesion kinase in cultured fibroblasts. We carried out yeast two-hybrid screening to identify cellular factors that form a complex with Hic-5 using its LIM domains as a bait, and we identified a cytoplasmic tyrosine phosphatase (PTP-PEST) as one of the partners of Hic-5. These two proteins are associated in mammalian cells. From in vitro binding experiments using deletion and point mutations, it was demonstrated that the essential domain in Hic-5 for the binding was LIM 3. As for PTP-PEST, one of the five proline-rich sequences found on PTP-PEST, Pro-2, was identified as the binding site for Hic-5 in in vitro binding assays. Paxillin also binds to the Pro-2 domain of PTP-PEST. In conclusion, Hic-5 may participate in the regulation of signaling cascade through its interaction with distinct tyrosine kinases and phosphatases.

hic-5 was isolated as one of transforming growth factor-β1- and hydrogen peroxide-inducible clones from mouse osteoblastic cells by a differential hybridization method and encodes a protein of 55 kDa (1). Its overexpression induced senescence-like phenotypes in immortalized fibroblasts (2) and promoted cellular differentiation process in cell lines such as osteoblasts (3). One of the unique features of the Hic-5 protein is its similarity to paxillin, especially in their C-terminal four LIM domains. Paxillin is a vinculin-binding protein co-localized with FAK1 and integrins in focal contacts (4). It is a phosphoprotein that interacts with tyrosine kinases of the Src family as well as with focal adhesion kinase and vinculin at focal adhesions (4).

The LIM domain is a unique cysteine-rich motif that defines a double zinc finger structure with a consensus sequence of (CXXCXX_16–23HXCCXCCX_16–23CXX/D/H/C) and is found in a variety of proteins with diverse functions and subcellular distributions, including transcription factors, components of adhesion plaques, and the actin-based cytoskeleton (5). The members of LIM proteins can be classified into five groups as follows: LIM homeodomain, LIM only protein, LIM kinase, GTPase-activating protein family, and zyxin family including enigma, paxillin, and Hic-5 (5). Accumulating evidence demonstrated that the LIM domains serves as an interface for protein-protein interactions (6), but the function of the LIM domains in the Hic-5 protein has not yet been determined.

Recent immunocytochemical studies showed that the Hic-5 protein is localized in focal adhesions in rat fibroblasts (cell strain WFB) and associates with cell adhesion kinase-β (7). Focal adhesions are specialized sites of cell adhesion to the extracellular matrix, consisting of the integrins, cytoskeletal proteins, and signaling molecules such as protein-tyrosine kinases and small G proteins (8–10). Integrins are the transmembrane extracellular matrix receptors (11), and the interaction of extracellular matrix and integrins elicits numerous cellular responses such as proliferation, migration, and differentiation (12–14). Formation of focal adhesions thus plays a central role in these biological responses (15).

Protein tyrosine phosphorylation plays an important role in the assembly of focal adhesions followed by transmission of extracellular stimuli (13), and protein-tyrosine kinases such as FAK (16), proline-rich kinase 2 (17), and cell adhesion kinase-β (18) are involved in the formation of focal adhesion complexes (19). Recently, it has been shown that protein-tyrosine phosphatases such as PTEN (20) and PTP-PEST (2) are also localized in the focal adhesion plaques. In the present communication, we describe evidence that PTP-PEST is associated with Hic-5 through its distinct LIM domains.

EXPERIMENTAL PROCEDURES

Screening of Factors That Associate with Hic-5—The cDNA library for expression of fusion proteins in yeast was constructed using mRNA from differentiated mouse myogenic cells, C2C12 (21). Total RNA was extracted from cells that were cultured for 4 days in a differentiation medium (Dubesco’s modified Eagle’s minimal essential medium supplemented with 2% horse serum) by the guanidinium/hot phenol method (1). Poly(A)+ RNA was isolated by dT30 latex (Oligotech™dT30, Takara Shuzo, Co., Kyoto, Japan). Double-stranded cDNA was synthesized by using the Two-hybrid cDNA Library Construction Kit (CLONTECH, Palo Alto, CA), according to the instruction manuals, and cloned into the EcoRI site of the pGAD10 vector (CLONTECH). As a bait, a cDNA fragment that encodes the LIM domain of Hic-5 (amino
acids 187–444) was ligated into BamHI site of pGBT9 to express a fusion protein with GAL4 DNA binding domain. By using the MATCH-MAKER two-hybrid system (CLONTECH), approximately $1 \times 10^{5}$ independent clones were screened, and cDNA clones that interacted with the Hic-5 LIM domains were selected by growth on His-deficient plates. Positive clones were further screened and LacZ expression in these clones were then allowed to test their two plasmid dependences. From $10^{6}$ his+ transformants, we obtained 16 interacting cDNA clones. These clones were sequenced using CySTM AutoRead® Sequencing Kit (Amersham Pharmacia Biotech Inc, Little Chalfont, UK) according to the manufacturer's instructions.

**Cell Culture and Transient Transfection of Plasmids—**Mouse myoblastic cells, C2C12, were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 15% fetal bovine serum and 50 μg/ml kanamycin under a humidified atmosphere of 5% CO₂ in air. Human immortalized fibroblasts, KMST-6 (42), were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were passaged at 1:8 dilution when they became confluent. Cells were transfected with plasmids using LipofectAMINE PLUS (Life Technologies, Inc.) according to manufacturer's manuals. Twenty-four hours post-transfection, cells were harvested and suspended in lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate) and incubated at 4 °C for 30 min. The insoluble material was then removed by centrifugation for 15 min at 20,000 × g, and the resultant lysates were analyzed by Western blotting and immunoprecipitation.

**Immunoprecipitation and Immunoblotting—**The anti-mouse Hic-5 polyclonal antibody (number 1024) was raised against the recombinant Hic-5 N-terminal region (amino acids 2–194). The monoclonal anti-phosphotyrosine antibody (PY20) and the anti-HA antibody (12CA5) were purchased from Transduction Laboratories (Lexington, KY) and Boehringer Mannheim, respectively. Immunoprecipitation of the HA-tagged proteins and the Hic-5 were performed after covalent coupling of antibodies to protein A- or protein G-beads (Pharmacia Biotech, Inc., Uppsala, Sweden) with the chemical cross-linking agent dimethyl pimelimidate (22). The covalently antibody beads were incubated for 16 h at 4 °C in the lysates. The immune complexes were collected by centrifugation, washed with the lysis buffer, and eluted with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol, 0.1 M dithiothreitol).

For immunoblotting, cell lysates and immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond C, Amersham Pharmacia Biotech). The membranes were blocked with bovine serum albumin and incubated with the antibodies or the anti-HA antibody for 16 h. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and a chemiluminescent detection kit using (ECL, Amersham Pharmacia Biotech).

**Plasmid Construction—**To generate Hic-5 deletion mutants (LIM 1–3, LIM 1–2, LIM 1, and ΔLIM), Hic-5 cDNA on the CMV/S5 vector (1) was used. Restriction enzymes XbaI and Sall were used for the restriction sites for constructing the ΔLIM plasmid. To introduce the β-galactosidase reporter into the Hic-5 mutants of the LIM domains (mLIM 1, mLIM 2, and mLIM 3) were excised from the pKF 19k vector and subcloned into the Pro-2 domain (HA-A-Pro-2) was generated by polymerase chain reaction. The PEP construct, PEP CL3/CMVScript, was provided by Dr. Rumi-nori Hasegawa (Department of Immunology, Tokyo Metropolitan Institute of Neuroscience, Kodaira, Tokyo, Japan).

GST Affinity Matrix Binding Assay—The fusion proteins were expressed by incubation of E. coli harboring expression vectors with 1 mM isopropyl–β-D-thiogalactopyranoside for 3 h at 30 °C. The bacteria were collected by centrifugation, lysed by sonication, and solubilized in phosphate-buffered saline containing 1 mM Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The GST fusion protein was expressed in E. coli strain DH5α.

**In Vivo Association of PTP-PEST and Hic-5 Mutants—**Flag-tagged Hic-5 mutants of the LIM domains (mLIM 1, mLIM 2, and mLIM 3) were inserted into pFLAG-CMV-2 vector (Eastman Kodak Co.) and co-transfected with HA-tagged PTP-PEST into KMST-6 human fibroblasts by calcium phosphate precipitation method. Cells were collected 24 h after transfection, and their association was analyzed by immunoprecipitation with anti-Flag and Western blotting with anti-HA as described above.

**Immunocytochemistry—**Cells were transfected with Flag-tagged Hic-5 mutants into KMST-6 cells by calcium phosphate method and fixed in 3.7% formaldehyde in phosphate-buffered saline 24 h after the transfection. Cells were permeabilized in 0.2% Triton X-100 in phosphate-buffered saline, treated with monconal anti-Flag antibody (Sig-
Isolation of cDNA Clones Encoding Hic-5-binding Proteins—To investigate the molecular basis of Hic-5-mediated cellular functions, we attempted to isolate interacting proteins of Hic-5 with the yeast two-hybrid system (26). As previously reported, the Hic-5 levels decreased in the most immortalized cell lines (1), but mouse myoblastic cells, C2C12, express significant amounts of Hic-5. The cDNA library was constructed with mRNA from C2C12 cells and screened using the LIM domain of Hic-5 as a bait. Sequences of 5 clones out of 16 positives were 100% identical to a part of a previously cloned cDNA, murine protein-tyrosine phosphatase-PEST (PTP-PEST) (27–30). The longest cDNA contained amino acids 185–433 and the shortest one 212–420 of PTP-PEST. It is a ubiquitously expressed protein-tyrosine phosphatase that is composed of an N-terminal phosphatase domain and a C-terminal noncatalytic region. The cDNA fragments isolated from the two-hybrid screening contained a part of a catalytic domain and proline-rich domains.

Hic-5 and PTP-PEST Associate in Vivo—To confirm that these two are associated in mammalian cells, we introduced expression vectors of HA-tagged PTP-PEST into the C2C12 cells, and we examined their binding by immunoprecipitation and Western blotting. The N-terminal HA-tagged PTP-PEST (29) or its mutant (C231S) in which a cysteine residue in the active center was converted to serine residue was transiently expressed in C2C12 cells, and then endogenous Hic-5 was immunoprecipitated with anti-Hic-5 antibody (number 1024). HA-PTP-PEST or HA-C231S was co-immunoprecipitated with Hic-5 (Fig. 1A), showing their in vivo association. In the immunoprecipitation using anti-HA antibody (12CA5), Hic-5 was co-immunoprecipitated with HA-PTP-PEST or HA-C231S (Fig. 1B). Surprisingly, the interaction of Hic-5 to wild type PTP-PEST and to mutant C231S were almost at similar levels. HA-C231S has a point mutation in the catalytic domain that can stably bind the substrates, but more investigation will be required to eliminate the possibility that Hic-5 is a substrate of PTP-PEST.

Domains of PTP-PEST for Interaction with Hic-5—One of the isolated clones of PTP-PEST encoded the amino acid residues 185–433 encompassing the C-terminal portion of the catalytic domain and the N-terminal region of the noncatalytic domain that includes Pro-1 and Pro-2 domains. In order to identify the domain responsible for interaction with Hic-5, we performed GST affinity matrix binding assay using a series of GST fusions of Pro-rich domains derived from PTP-PEST (Fig. 2A). These domains have been shown to provide the interface for interacting with Src homology domain 3-containing signal transduction molecules. In order to identify the domain responsible for association with Hic-5, we performed GST affinity matrix binding assay using a series of GST fusions of Pro-rich domains derived from PTP-PEST (Fig. 2A). The fusion proteins were immobilized on glutathione beads and incubated with
35S-labeled Hic-5 produced by in vitro translation. The proteins bound to the beads were eluted, separated by SDS-PAGE, and exposed to x-ray film. The result demonstrated that the Hic-5 preferentially binds to the GST-Pro-2 domain, whereas Pro-1, and Pro-3–5 showed essentially no binding activity (Fig. 2, B and C). A fusion protein of the Pro-2 domain showed unusual mobility on SDS-PAGE, but it is generally accepted that Pro-rich peptides migrate unpredictably. Furthermore, all constructs have been sequenced, and we are sure that they encode for the proper proteins (36). Paxillin also bound to the Pro-2 domain of PTP-PEST.

**Determination of the LIM Domains Essential for Interaction with PTP-PEST**—The Hic-5 C-terminal region used as a bait contains four LIM domains. Because a single LIM domain can act as a protein association interface (5), we tried to map the LIM domain(s) required for binding to PTP-PEST. The binding assay was carried out using GST fusion protein of PTP-PEST isolated from two-hybrid screening and 35S-labeled Hic-5 deletion mutants. Since both LIM 1–3 and LIM 3–4 could bind to PTP-PEST, LIM 1, LIM 2, and LIM 4 are not necessary for the association (Fig. 3, A and B). These data indicate that LIM 3 of Hic-5 is important for interaction with PTP-PEST. However, the Hic-5 mutant containing only LIM 3 could not bind to PTP-PEST (Fig. 3B), suggesting that LIM 3 alone is not sufficient for the binding. It is most likely that each LIM may affect the structural stability of adjacent LIMs or functions cooperatively.

To ascertain the requirement of LIM 3 for binding to PTP-PEST, we introduced two point mutations in LIM 2 or LIM 3 by site-directed mutagenesis. A single LIM motif is composed of two zinc fingers with the following consensus sequence: CX2CX16–23HX2CX2CX16–21CX2(C/H/D). Each zinc finger is folded into its ternary structure chelating a zinc ion via conserved cysteine, histidine, or aspartate residues, and the introduction of point mutations in zinc-chelating residues can disrupt the ternary folding of LIM structure (31). The mutants used here, mLIM 2 (H293L/C296G) and mLIM 3 (H352G/C355G), contained point mutations at zinc-chelating residues within both the first and second zinc fingers, disrupting the structure of an individual LIM domain. We performed the binding assay using these mutants and a GST fusion Pro-2 domain of PTP-PEST as a GST matrix (Fig. 3C). In contrast to the mLIM 2 protein that bound to PTP-PEST efficiently, mLIM 3 did not show any binding activity. This indicates that the LIM 3 domain of Hic-5 is essential to associate with PTP-PEST. Taken together with the above results, we concluded that Hic-5 and PTP-PEST associate through LIM 3 domain and Pro-2 domain, respectively, in vitro.

We also examined the specificity of interaction between Hic-5 and PTP-PEST by comparing to another protein-tyrosine phosphatase, PEP (32). PEP is a nonreceptor type PTP with similar structure to PTP-PEST, and we performed in vitro binding assay using a GST fusion protein of the LIM domains of Hic-5 and 35S-labeled PEP. LIM domains of Hic-5 failed to bind to PEP (data not shown), suggesting that these LIM domains have binding selectivity to a certain type of PTPs.
In Vivo Association between Mutants of Hic-5 and PTP-PEST—The above results show that LIM 3 of Hic-5 and Pro-2 of PTP-PEST specifically bind each other in vitro, but their in vivo association was examined by transfection of each expression vectors. In Fig. 4A, plasmids that express HA-tagged full-length PTP-PEST or its Pro-2 deletion mutant (ΔPro-2) were introduced into C2C12 cells. Cell lysates were treated with anti-Hic-5 to precipitate endogenous Hic-5, and the precipitates were Western-blotted with anti-HA antibody. The results clearly indicate that Pro-2 deletion mutant did not associate with Hic-5. Next we transfected human fibroblastic KMST-6 cells with Flag-tagged Hic-5 mutants together with HA-PTP-PEST. This cell line was chosen, because of its low level of endogenous Hic-5 that would interfere with interaction of exogenously expressed products. Cellular lysates were immunoprecipitated with anti-HA and Western-blotted with anti-Hic-5 antibody. In contrast to the results obtained from in vitro binding assay, the results shown in Fig. 4B indicate that Hic-5 with point mutation either in LIM 1, LIM 2, or LIM 3 formed a complex with HA-tagged PTP-PEST. This may result from the presence of other factors that bridge between Hic-5 and PTP-PEST through other domains.

Subcellular Localization of the LIM Mutants—It is reported that the LIM 3 of paxillin determines its localization to focal adhesion (38), and subcellular localization of Hic-5 with mutations at the LIM domains was examined using Flag-tagged Hic-5 expressing either wild type or mutated LIMs. KMST-6 cells were transfected with pRC/CMV-based expression vectors, and cells were fixed 24 h later for immunostaining with anti-Flag antibody. In Fig. 5, A–D, the results show that wild type Hic-5 localized in focal adhesion, but the mutant in LIM 3 completely lost this phenotype (Fig. 5D). Localization of the mutants at LIM 1 and LIM 2 in focal adhesion seemed weakened (Fig. 5, B and C) compared with that of wild type Hic-5.

Involvement of PTP-PEST for subcellular localization of Hic-5 was tested using fibroblasts from PTP-PEST(+/−) and (−/−) mice. An expression vector of HA-tagged full-length PTP-PEST was introduced into embryo fibroblasts, and localization of Hic-5 was observed by staining with anti-HA antibody. Comparison of Fig. 5, E and F, indicates that Hic-5 localized in focal adhesion both in PTP-PEST(+/−) and (−/−) cells, but Hic-5 seems mainly localize in stress fibers in (−/−) cells.

DISCUSSION

Hic-5, a member of LIM proteins, has been shown to be involved in cellular senescence and differentiation processes (2, 3). However, the molecular mechanisms for its biological effects have been remained unsolved, whereas its localization in focal adhesion (7) suggested to us that Hic-5 has some roles in modulating integrin-mediated signal. In this study, we isolated the Hic-5 interacting factors using a yeast two-hybrid system and identified PTP-PEST/P19-PTP, which is a member of the intracellular PTP family, as one of such factors.

Protein tyrosine phosphorylation is one of the most important entities constituting signal transduction pathways from cell surface to the nucleus in response to various extracellular stimuli (33). The level of protein tyrosine phosphorylation increases not only upon stimuli with soluble factors, such as growth factors, but also upon cell adhesion to extracellular substrate (34). Many molecules are now known to be tyrosine-phosphorylated upon cell adhesion events and thought to function in transmitting the signal emanating from integrins, which have no intrinsic tyrosine-phosphorylating activity, to the nucleus (reviewed in Refs. 12–15). For example, paxillin and FAK are tyrosine-phosphorylated following stimulation of integrin signals (19, 34). Protein tyrosine phosphorylation is so critical that its level should be tightly regulated by the balanced activities of protein-tyrosine kinases and phosphatases. Thus, like protein-tyrosine kinases, PTPs are important molecules in controlling the level of protein tyrosine phosphorylation.

PTP-PEST (also known as P19-PTP) is a member of the intracellular PTP family and is characterized by the presence of several PEST motifs in the carboxyl segment of the protein adjacent to the catalytic domain (26–29). In addition, its C terminus contains five proline-rich segments (29). At least two of these domains represent type 2 proline-rich consensus segments known to interact with Src homology domain 3 motif. To date, several molecules have been identified to interact with PTP-PEST, including adaptor proteins such as SHC (35) and...
Grb2 (24). The recent study using fibroblasts derived from PTP-PEST knock-out mice has identified p130Cas as one of the downstream targets of PTP-PEST (36). Cas is localized in focal adhesions (37), as well as PTP-PEST following cell adhesion to fibronectin and concomitant engagement of integrins (2). It is plausible that the PTP-PEST interacting factors including Hic-5 modulate such PTP-PEST activity or substrate specificity, thereby contributing to signal transduction in focal adhesions.

From the series of experiments using various types of mutant proteins, we concluded that Hic-5 interacts with the Pro-2 domain of PTP-PEST by LIM 3 in vitro as summarized in Fig. 6. On the other hand, the results obtained from in vitro binding assay shown in Fig. 4B indicate that Hic-5 with point mutation either in LIM 1, LIM 2, or LIM 3 formed a complex with HA-tagged PTP-PEST. This may result from the presence of another factor that bridges between Hic-5 and PTP-PEST through other domains.

The LIM domains of Hic-5 are very similar to those of paxillin, and overall sequence similarity of amino acids in the LIM domains of Hic-5 and paxillin is 68%. In particular, similarity is as high as 74% in LIM 3. Therefore LIM 3 domains of both proteins possibly bear the similar function. We have shown that the LIM 3 of Hic-5 is the binding site for PTP-PEST, and the LIM 3 of paxillin is known to participate in localization of the protein to focal adhesion (38). Besides, both Hic-5 and paxillin are actually localized at focal adhesions and bind to PTP-PEST. Taken together, an interesting possibility is raised that LIM 3 is the determinant of subcellular localization of Hic-5 and paxillin and that PTP-PEST participates in recruiting them to focal adhesions by binding to LIM 3 of both proteins. The results of Fig. 5, A–D, however, suggest that the LIM domains other than LIM 3 may be involved in localization in focal adhesion. These possibilities are an open question for future study.

N-terminal regions of Hic-5 and paxillin are also very similar in structure, although their similarity is less than in LIM domains. The regions contain Pro-rich and LD domains, which are thought to serve as an interface for protein-protein interaction. In fact FAK, proline-rich kinase 2, and vinculin have been shown to bind to the N-terminal domains of paxillin (39), and Fujita et al. (40) recently reported that Hic-5 associates with FAK at the N-terminal domain. Collectively, Hic-5 and paxillin are closely related proteins in many aspects, such as structures, interacting factors, and subcellular localization. However, they are expected to have different functions within cells, since their expression patterns are different; paxillin is ubiquitously expressed, whereas Hic-5 is expressed higher in spleen and lung (1). At molecular levels, Fujita et al. (40) have clearly demonstrated the differences between Hic-5 and paxillin. They have shown that Hic-5 is marginally tyrosine-phosphorylated either by FAK kinase activity or by integrin stimulation, which forms sharp contrast to paxillin that is heavily phosphorylated by either pathway. This result is consistent with the fact that Hic-5 does not have several tyrosine residues that are present in paxillin and are supposed to be binding sites for the Crk SH2 domain and potentially phosphorylated (40). Of interest is that fibronectin-induced tyrosine phosphorylation of paxillin was inhibited by co-expression of Hic-5 (40). From these observations, they proposed that one of putative proteins is...
Hic-5 functions is to inhibit tyrosine phosphorylation of paxillin by binding to protein-tyrosine kinases such as FAK in a competitive manner to paxillin. Recent findings by Shen et al. (41) indicated that paxillin associates with PTP-PEST through the region of 297–494 amino acids encompassing Pro-1 and Pro-2. Although the precise mapping of the interacting domain of PTP-PEST with paxillin has not been yet performed, it is likely that Hic-5 binding to Pro-2 competes out paxillin, resulting in inhibition of the signaling directed by the complex of PTP-PEST and paxillin as is the case of protein-tyrosine kinases.

In conclusion Hic-5 shares structural features, subcellular localization, and interacting factors with paxillin. FAK and PYK2 have been reported to bind to both Hic-5 and paxillin. In this study we added PTP-PEST to the list. All of these molecules are implicated in protein tyrosine phosphorylation and are believed to have important functions in integrin-mediated signal transduction pathway. Hic-5 is thought to compete with paxillin in the binding to these molecules and thus control tyrosine phosphorylation signaling mediated by paxillin. Further study on Hic-5 functions is expected to clarify the nature of integrin-mediated signaling in more detail and the way it is involved in various types of cellular processes.

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