Hic-5, of Proteins Localized at Focal Adhesions*

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Hic-5 associated with CAK

Cell adhesion kinase β (CAKβ/PYK2) is the second protein-tyrosine kinase of the focal adhesion kinase subfamily. We identified a cDNA that encodes a CAKβ-binding protein. This cDNA clone encodes the human homologue of Hic-5, the cDNA of which was cloned in 1994 as transforming growth factor β1- and hydrogen peroxide-inducible mRNA. We found that Hic-5 exclusively localized at focal adhesions in a rat fibroblast line, WFB. This localization of Hic-5 was confirmed in WFB cells expressing Myc-tagged Hic-5. The amino acid sequence of Hic-5 is highly similar to that of paxillin in the four LD motifs as well as in the four contiguous LIM domains.

The Hic-5 N-terminal domain directly associated in vitro with the extreme C-terminal region (residue 801 to the end) of CAKβ. CAKβ was coimmunoprecipitated with Hic-5 from the WFB cell lysate. The coimmunoprecipitation of CAKβ with Hic-5 was markedly inhibited by the addition of the extreme C-terminal region of CAKβ. Coimmunoprecipitation of Hic-5 with CAKβ, which was shown in COS-7 cells doubly transfected with cDNA constructs of CAKβ and Myc-tagged Hic-5, was lost when the CAKβ amino acid residues 741–903 were deleted. Hic-5 was tyrosine-phosphorylated in Src-transfected 3Y1 cells and in cells treated with pervanadate. Hic-5 associated with CAKβ was selectively tyrosine-phosphorylated in WFB cells exposed to hypertonic osmotic stress. These results indicate that Hic-5 is a paxillin-related component of focal adhesions and binds to CAKβ, implying possible involvement of Hic-5 in the downstream signaling of CAKβ.

We found, by cDNA cloning, the second protein-tyrosine kinase of the focal adhesion kinase subfamily. This paper is available online at http://www.jbc.org

1 The abbreviations used are: FAK, focal adhesion kinase; CAKβ, cell adhesion kinase β; N-domain, N-terminal domain; C-domain, C-terminal domain; SH2, Src homology domain 2; SH3, Src homology domain 3; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ECL, enzyme-linked chemiluminescence; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PBS1 and PBS2, paxillin-binding subdomains 1 and 2; bp, base pair(s); HSV, herpes simplex virus.

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cells are stressed by osmotic shock (5, 22). The differences in the subcellular and tissue distributions of CAKβ and FAK indicate different functions for these two protein-tyrosine kinases.

In a study to elucidate the upstream and downstream signaling pathways of CAKβ, we used an expression cloning technique to identify binding partners for the C-domain of CAKβ. We report here the identification of a cDNA that encodes a CAKβ-binding protein. The predicted amino acid sequence of this cDNA indicated that the protein was the human homologue of Hic-5, the cDNA of which was described by Shibahama et al. (23) as a transforming growth factor β1- and hydrogen peroxide-inducible mRNA. Hic-5 is closely related to paxillin in its amino acid sequence. Immunocytochemical staining of rat fibroblast line WFB with a specific anti-Hic-5 antibody revealed that Hic-5 localized exclusively at focal adhesions as paxillin did, but in disagreement with the original identification of Hic-5 as a nuclear protein (23, 24). We further demonstrated communoprecipitation as well as direct binding of Hic-5 and CAKβ. Hic-5 associated with CAKβ was preferentially tyrosine-phosphorylated, implying a functional interplay between CAKβ and Hic-5.

MATERIALS AND METHODS

Cloning of a cDNA Encoding a CAKβ-Binding Protein—An oligo(dT)-and random Primed human (normal female, 2 years old) hippocampal cDNA library constructed in λZAPII vector (Stratagene, La Jolla, CA) was screened by affinity binding to a glutathione-Sepharose 4B. The GST fusion protein was expressed in Escherichia coli strain BL21(DE3), affinity-purified by using glutathione-agarose, and phosphorylated in vitro using the catalytic subunit of cAMP-dependent protein kinase (Sigma) and [γ-32P]ATP (ICN Biochemicals, Inc., CA) as described by Hildebrandt et al. (15) and Kaelin et al. (25).

Screening of λZAPII expression libraries was done as described below. Phage plaques were formed on culture plates at 37 °C, and the protein expression was induced by β-mercaptoethanol (β-ME) (15B5; Schleicher & Schuell) that had been soaked in 10 mM isopropryl-β-D-thiogalactopyranoside. After overnight incubation at 37 °C, the membranes were removed and washed twice at 4 °C in Hyb75 buffer (20 mM HEPES (pH 7.5), 55 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl2, 1 mM dithiothreitol, 0.05% Nonidet P-40) (25). The membranes were soaked twice for 10 min in Hyb75 buffer supplemented with 6 μg/ml proteinase K at 37 °C (in fresh solution) and then for 5 min in Hyb75 buffer supplemented with 3 μg/ml ribonuclease A and 0.75 μg/ml proteinase K. This order. After soaking in Hyb75 buffer containing 5% skim milk at 4 °C for 1 h with constant shaking, which represents a blocking step, the membranes were then incubated with 106 cpm/ml of the 32P-labeled GST-CAKβ-Cdom (50 ng/ml) in Hyb75 buffer, which contained 1% skim milk and 200 μg/ml of GST-proliferated by expression from pGEX-2TK vector. The membranes were washed seven times for 15 min each in Hyb75 buffer containing 1% skim milk. Positive plaques were made visible by exposure of the membranes to X-ray films. The positive clone thus obtained, cbp-1, was subcloned into pBluescript and subjected to sequencing in both directions after the preparation of internal deletion mutants.

Epitope-Tagged Hic-5 and Fusion Proteins—The plasmid construct encoding the N-terminally Myc-tagged Hic-5 was generated as follows. Using polymerase chain reaction, the cDNA encoding full-length (according to the open reading frame described by Shibahama et al. (23)) human Hic-5 was amplified, and BamHI and EcoRI restriction sites were created at nucleotide positions +6 (immediate 5′-side of the presumptive translational initiation codon ATG) and +1492, respectively. The amplified cDNA was ligated in frame to the BamHI and EcoRI sites of the pcDNA3Myc vector to obtain pHiic5-Myc. The pcDNA3Myc vector was constructed by ligating the HindIII-BglII fragment of the pJ3M vector (26) into the HindIII and BamHI sites of pcDNA3 (Invitrogen). The 10 amino acid residues of the epitope tag are specifically recognized by the anti-Myc monoclonal antibody 9E10.

pGEX-Hic5(f) encoding the fusion protein GST-Hic5(f) was constructed by cloning whole Hic-5 cDNA sequences of cbp-1 including those of the presumed 5′-noncoding region (23) into the EcoRI site of pGEX-3X. For expression of GST-Hic5-Ndom and GST-Hic5-Cdom, clone cbp-1 cDNA sequences encoding 223 N-terminal amino acid residues (668–928) (23) were amplified from cDNA encoding 223 amino acid residues (668–1009) (23) were amplified from cDNA encoding 223 amino acid residues (668–1009) (23) and cloned into pGEX-2TK. GST-CAKβ-Ndom, GST-CAKβ-Cdom, GST-CAKβ-CdomA, and GST-CAKβ-CdomB were engineered by cloning rat CAKβ cDNA sequences encoding residues 5 to 416, 670 to 1009, 670 to 792, and 801 to 1009, respectively, into pGEX-2TK. For expression of GST-FAK-Cdom, GST-FAK-CdomA, and GST-FAK-CdomB, mouse FAK cDNA sequences encoding 305 amino acid residues (668–1009), 123 amino acid residues (668–790), and 257 amino acid residues (765–1009), respectively, of the C-domain were amplified by polymerase chain reaction from clone pBSFAK and cloned into pGEX-2TK. pBSFAK was kindly provided by Dr. S. K. Hanks (27). The GST fusion proteins were expressed in E. coli strain BL21(DE3) and affinity-purified by using glutathione-agarose (Sigma) (28).

For the construction of the plasmid encoding the GST fusion protein of full-length CAKβ (GST-CAKβ(fl)), a rat CAKβ cDNA with a BamHI site 9 base pairs upstream from the translational initiation codon of CAKβ was first prepared by the use of polymerase chain reaction and rejoining of cDNA fragments. The BamHI-EcoRI fragment, containing the whole coding region of CAKβ cDNA, was prepared, and its 3′-flank was removed by treatment with DNA polymerase I (Klenow fragment). This fragment was ligated into pEBG-Sma vector at the BamHI and SmaI sites. pEBG-Sma was prepared by ligating SmaI linker to the pEBG vector (29). The plasmid thus prepared, pEBG-CAKβ, was transfected into 293 cells grown on 15-cm dishes at 20 μg/dish by the method of calcium phosphate coprecipitation. Two days after the transfection, the cells were lysed, and GST-CAKβ(fl) was purified by binding to glutathione-agarose.

Deletion Mutants of CAKβ—The full-length CAKβ cDNA clone, 17N, and the C-terminally epitope-tagged CAKβ cDNA were subcloned into expression vector pSIRE to obtain pCAKβ(S) and pCAKβTag as described previously (1). pCAKβ(S) and pCAKβTag were used for the generation of CAKβ variants. Deletion (dl) mutations are designated by the amino acid residues deleted. The base pair (bp) designation corresponds to the nucleotide sequence of the CAKβ cDNA counting from the translational initiation codon (1). Mutation dl 86–321 was generated by digesting pCAKβTag with PvuII (which cleaves at bp 253, 529, 862, 961, and 2689) and isolating the largest fragment and the fragment of 1737 base pairs followed by rejoining of the PvuII termini; rejoining of the 1737-bp fragment in the right direction was confirmed. To construct dl 159–552, pCAKβTag was digested with restriction enzymes (BspEI, 680, and 1655) followed by religation of the BspEI end. This dl 159–552 mutant of pCAKβ(S) was digested with SacI (which cleaves at bp 2854) and then the SacI fragment of pCAKβTag containing the Tag cDNA sequences was ligated into the SacI site of the deletion mutant of pCAKβ(S) to generate the dl 159–552 mutant of pCAKβTag. Mutation dl 741–903 was created by digesting pCAKβTag with BsmI (which cleaves at bp 2218 and 2707) followed by religation of the BsmI termini.

Production of Antiserum to Hic-5 and Affinity Purification of the Antibody—The anti-Hic-5 antibody was raised in rabbits against a GST fusion protein of full-length human Hic-5, GST-Hic5(fl). Anti-GST antibody was first removed from the serum by the use of a column of covalently bound GST. Anti-Hic-5 was then affinity-purified on a column of the immunogen covalently bound to cyanogen bromide-activated Sepharose 4B, from which the antibody was eluted with 0.5 M ammonium hydroxide containing 3 M sodium thiocyanate (pH 11.0). The anti-Hic-5 antibody immunoprecipitated Hic-5 of human and rat origin and was good for use in immunoblotting and immunocytochemistry.

Antibodies and Other Materials—The first anti-CAKβ rabbit antibody used in this study, anti-CAKβ(C-a), was raised against a GST fusion protein of residues 670–716 of rat CAKβ and was affinity-purified on a column of the immunogen covalently bound to cyanogen bromide-activated Sepharose 4B, from which the antibody was eluted with 0.5 M ammonium hydroxide containing 3 M sodium thiocyanate (pH 11.0). The anti-Hic-5 antibody immunoprecipitated Hic-5 of human and rat origin and was used in immunoblotting or after removal of anti-GST followed by affinity purification on a column of immobilized immunogen. Anti-CAKβ(N) was also found to be specific to CAKβ. Anti-CAKβ(N)-mAb and anti-CAKβ(C)-mAb are mouse IgG1, monoclonal antibodies raised against GST-CAKβ-Ndom and GST-CAKβ-Cdom, respectively.

Fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulins
and rhodamine-conjugated anti-mouse immunoglobulins were purchased from DAKO (Copenhagen, Denmark). Monoclonal anti-FAK (clone 77, mouse IgG1) and anti-paxillin (clone 349, mouse IgG1) antibodies were obtained from Transduction Laboratories (Lexington, KY). Monoclonal anti-rabbit immunoglobulin (clone RG-16, which reacts with mouse IgG and IgG1) (catalog number was 1005-1005b (N)) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Myc monoclonal antibody 9E10 (IgG1) was obtained from the culture supernatant of MYC 1–9E10.2 cells (ATCC, CRL 1729) and purified by binding to a protein A column (Ampure PA kit, Amersham Corp.). Affinity-purified anti-GST rabbit antibody was a byproduct obtained from the GST protein A column (Ampure PA kit, Amersham Corp.). Affinity-purified anti-GST rabbit antibody was by a procedure obtained from the GST column step in the purification of anti-Hic-5 and anti-CAKβ.

**Cells**—A rat fibroblast line, WFB (30), was obtained from the supplier of the line, Dr. N. Sato (Sapporo Medical University, Sapporo, Japan). A rat fibroblast line transformed with Rous sarcoma virus, SR-3Y1-1 (SR-3Y1, RCB0353) (31), and its parent line, 3Y1-B clone 1–6 (3Y1, RCB0488) (32), were obtained from Riken Cell Bank (Tsukuba, Japan). COS-7 (ATCC ARLC 1651) and HER 293 (CRL 1573) were obtained from the American Type Culture Collection (Rockville, MD). These cells were cultured in Iscove’s modified Dulbecco’s medium (Iscove’s) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin. When WFB cells were stimulated, the medium of confluent monolayer cultures of the cells in 10-cm dishes was changed with 2 ml of warm Iscove’s medium without serum. After 1 h of incubation at 37 °C, the cells were stimulated at 37 °C for 3-10 min with either 2 μM oleyl l-α-lysophosphatidic acid (Sigma) or 0.2 μM endothelin 1 (Sigma) or exposed to hypotonic medium at 37 °C for 5-20 min by replacing the medium with 2 ml of warm Iscove’s medium containing 0.3 M sorbitol.

**Immunofluorescence Microscopy and Confocal Laser-scanning Microscopy**—Cells grown on glass coverslips coated with rat tail collagen (33) were fixed with cold absolute ethanol unless otherwise stated and kept at −20 °C until use. After being rinsed with phosphate-buffered saline (PBS), the cells were incubated with Block Ace (Dainippon Pharmaceutical Co., Tokyo, Japan) at room temperature for 30 min. Then a primary antibody was applied. The incubation with anti-Hic-5 antibodies was done for 1 h at room temperature, and the incubation with other antibodies was done for 30 min at room temperature. Fluorescein isothiocyanate- or rhodamine-conjugated antibodies were then applied for 30 min at room temperature. The cells were thoroughly washed in PBS and incubated with the secondary antibody. After being rinsed with PBS, the coverslips were mounted in a solution of 10% PBS and 90% glycerol. Monoclonal anti-rabbit immunoglobulin (clone RG-16, which reacts with mouse IgG and IgG1) was obtained from the culture supernatant of MYC 1–9E10.2 cells (ATCC, CRL 1729) and purified by binding to a protein A column (Ampure PA kit, Amersham Corp.). Affinity-purified anti-GST rabbit antibody was a byproduct obtained from the GST column step in the purification of anti-Hic-5 and anti-CAKβ.

**RESULTS**

**Isolation of a cDNA Clone Encoding a Binding Protein to the CAKβ C-domain**—In an effort to identify proteins that bind CAKβ, we used the N- and C-domains of CAKβ, which are the regions contiguous to the kinase domain at the N- and C-terminal sides, to screen a ZAPII expression library derived from human hippocampus cDNA. One clone (clone cbp-1) was positively identified by screening of 2 × 10⁸ plaques with the C-domain, GST-CAKβ-Cdom (consisting of amino acid residues 670–1009 of rat CAKβ), as a probe. A comparison of 1759 base pairs of the cbp-1 cDNA sequence with those in the GenBank™ data base by the BLASTx program (35) of NCBI revealed high similarity of the amino acid sequence translated from cbp-1 in one reading frame and the Hic-5 amino acid sequence (GenBank™ L22482) over their entire length. This result indicated that cbp-1 is a cDNA clone encoding the full-length, human homologue of Hic-5, which had been cloned from a mouse cDNA library as a transforming growth factor β1- and hydrogen peroxide-inducible mRNA by Shibahara et al. (23). Human Hic-5 has the same number of amino acid residues as mouse Hic-5, and their amino acid sequences are 97.0% identical in their double zinc finger LIM domains (amino acid residues 211–444 (23)) and 96.37% identical in their N-domain (amino acid residues 1–210 (23)). The Hic-5 cDNA contains flanking 5'- and 3'-untranslated sequences of 49 and 376 base pairs, respectively. In the fusion protein encoded by clone cbp-1, the 49 base pairs at the presumed 5'-untranslated region (23) predicted 16-amino acid residues contiguous to β-galactosidase encoded by the phage vector used in the construction of the cDNA library.

A homology search in GenBank™ using the BLASTx program revealed that the amino acid sequence of Hic-5 had high similarity with that of paxillin both in its LIM domains and in
mouse cDNA cloned by Shibanuma pairs of human Hic-5 cDNA are totally different from those of between Hic-5 and paxillin strongly suggests that the 16 amino acid residues encoded by the presumed 5 motif corresponding to the first one in paxillin. However, the 16 motifs. Three LD motifs are identified in Hic-5 lacking the LD region contiguous to LD motifs (38) (Fig. 1). Paxillin has four LD motifs (38) (Fig. 1). Comparison of amino acid sequences of Hic-5 and paxillin. The sequence of human Hic-5 was deduced from the nucleotide sequence of human Hic-5 cDNA. However, we found only one more Hic-5 cDNA identified by Shibanuma (40). The amino acid residues of human Hic-5 were tentatively numbered according to the numbering by Shibanuma et al. (23), is not the true translational initiation site of the human Hic-5 (G). For this reason, the amino acid residues of human Hic-5 were tentatively numbered according to the numbering by Shibanuma et al. (23) (Fig. 1).<ref>FIG. 1.</ref> Its regions of LD motifs (38) (Fig. 1). Paxillin has four LD motifs. Three LD motifs are identified in Hic-5 lacking the LD motif corresponding to the first one in paxillin. However, the 16 amino acid residues encoded by the presumed 5-untranslated region contiguous to β-galactosidase were highly similar to the first LD motif of paxillin (Fig. 1). This sequence similarity between Hic-5 and paxillin strongly suggests that the 16 amino acid residues encoded by the presumed 5-untranslated region are, in fact, a part of the Hic-5 amino acid sequence and that the methionine at the residue 1 of Hic-5, according to the numbering by Shibanuma et al. (23), is not the true translational initiation site. The nucleotide sequences around this amino acid residue 1 fit poorly to the sequence context for translational initiation defined by Kozak (39), with neither purine at position –3 nor a G at position +4 (see the nucleotide sequences submitted to data bases). The nucleotide sequence of human Hic-5 cDNA is highly similar to that of mouse Hic-5 cDNA up to the 39 base pairs upstream of the presumed translational initiation codon, but the remaining 5′-terminal 10 base pairs of human Hic-5 cDNA are totally different from those of mouse cDNA cloned by Shibanuma et al. (23). We tried to identify the true translational initiation site of the human Hic-5 cDNA. However, we found only one more Hic-5 cDNA clone in the cDNA library where cbp-1 was cloned after an extensive screening with a fragment of cbp-1 as a probe and by nested polymerase chain reactions. This cDNA clone was 9 base pairs shorter at the 5′-end than the clone cbp-1. For this reason, the amino acid residues of human Hic-5 were tentatively numbered according to the numbering by Shibanuma et al. (23) (Fig. 1). Hic-5 and paxillin contain four contiguous double zinc finger LIM domains at their carboxyl-terminal halves, amino acid residues 211–444 of Hic-5, and 324–557 of paxillin. In the four LIM domains, the amino acid sequences of the two proteins are 62% identical. In the Hic-5 N-domain, amino acid sequence similarity with paxillin is limited to the LD motifs. Hic-5 has a proline-rich sequence at amino acid residues 14–20, which is a potential ligand to SH3 domains.

Hic-5 Localizes at Focal Adhesions—An anti-Hic-5 antibody was raised in rabbits against the GST fusion protein of human Hic-5 (GST-Hic5(fl)) and affinity-purified on a column of immobilized immunogen. The anti-Hic-5 antibody was found to be specific to Hic-5 as shown by immunoblotting and immunoprecipitation from the rat fibroblast WFB cell lysate, where a band of about 55 kDa was detected by the antibody (Fig. 2). The anti-paxillin monoclonal antibody obtained from Transduction Laboratories immunoprecipitated and immunoblotted not only paxillin but also Hic-5 (Fig. 2). The anti-Hic-5 antibody neither immunoprecipitated paxillin nor bound to paxillin blotted from gel after immunoprecipitation with anti-paxillin and separation by SDS-PAGE (Fig. 2).

This anti-Hic-5 antibody specifically immunostained focal adhesions in WFB cells with anti-Hic-5, The WFB cell lysate was prepared in a lysis buffer containing 0.5% sodium deoxycholate and 0.1% SDS in addition to 1% Nonidet P-40. Hic-5 was immunoprecipitated from 1.2 mg of protein of the lysate with 1 µg of protein of affinity-purified anti-Hic-5 bound to protein A-Sepharose (10 µl of packed volume). Paxillin was immunoprecipitated from 0.4 mg of protein of the lysate with 1 µg of protein of anti-paxillin monoclonal antibody bound to anti-mouse IgG-agarose (7.5 µl of packed volume). The immunoprecipitates and 60 µg of protein of the WFB cell lysate (total lysate) were subjected to SDS-PAGE in a 10% gel. The separated proteins were blotted onto a PVDF membrane. Anti-Hic5 antibody and anti-paxillin monoclonal antibody were used for immunoblotting, which is indicated at the bottom of each lane. Positions of molecular mass markers (Sigma SDS-7B) are indicated on the right, i.p., immunoprecipitation.

FIG. 2. Immunoprecipitation and immunoblotting of Hic-5 in WFB cells with anti-Hic-5. The WFB cell lysate was prepared in a lysis buffer containing 0.5% sodium deoxycholate and 0.1% SDS in addition to 1% Nonidet P-40. Hic-5 was immunoprecipitated from 1.2 mg of protein of the lysate with 1 µg of protein of affinity-purified anti-Hic-5 bound to protein A-Sepharose (10 µl of packed volume). Paxillin was immunoprecipitated from 0.4 mg of protein of the lysate with 1 µg of protein of anti-paxillin monoclonal antibody bound to anti-mouse IgG-agarose (7.5 µl of packed volume). The immunoprecipitates and 60 µg of protein of the WFB cell lysate (total lysate) were subjected to SDS-PAGE in a 10% gel. The separated proteins were blotted onto a PVDF membrane. Anti-Hic5 antibody and anti-paxillin monoclonal antibody were used for immunoblotting, which is indicated at the bottom of each lane. Positions of molecular mass markers (Sigma SDS-7B) are indicated on the right, i.p., immunoprecipitation.

Hic-5 forms a complex with Hic-5, a focal adhesion protein
the tagged Hic-5 was transiently expressed in WFB cells. The presence of the tagged Hic-5 at focal adhesions in transfected WFB cells is shown in Fig. 4 by immunostaining with anti-Myc. WFB cells grown on glass coverslips coated with rat tail collagen were doubly stained with anti-Hic-5 and anti-FAK. Confocal laser-scanning micrographs of the cells are shown for staining of Hic-5 (green in A), FAK (red in B), and both Hic-5 and FAK (yellow in D). A transmitted light phase-contrast micrograph of the same cell (C) is also shown. Colocalization of Hic-5 and FAK was visualized as yellow signals in D. The bar represents 10 μm. Double staining of Hic-5 and stress fibers. WFB cells grown on glass coverslips coated with rat tail collagen were doubly stained with anti-Hic-5 and rhodamine-conjugated phalloidin. Confocal laser-scanning micrographs of the cells are shown for staining of Hic-5 (green in A), stress fibers (red in B), and both Hic-5 and stress fibers (yellow in D). A transmitted light phase-contrast micrograph of the same cell (C) is also shown. Colocalization of Hic-5 and microfilaments was visualized as yellow signals in D. Both ends of stress fibers terminate at focal contacts where Hic-5 localizes. The bar represents 10 μm.
(amino acid residues 1–223; GST-Hic5-Ndom), the Hic-5 C-domain (amino acid residues 203–444 containing the LIM domains; GST-Hic5-Cdom), and, as a control, GST were subjected to SDS-PAGE and immobilized on a PVDF membrane. Hic-5 in the WFB cell lysate was immunoprecipitated with anti-Hic-5 and was also immobilized on the membrane after the electrophoretic separation. After procedures for denaturation and renaturation of the proteins on the membrane, the fusion proteins and Hic-5 were probed for binding to CAKβ and FAK. The probes used for the binding assay were 32P-labeled GST fusion proteins of the N- and C-domains of CAKβ, GST-CAKβ-Ndom and GST-CAKβ-Cdom; fragments of the C-domain, GST-CAKβ-CdomA and GST-CAKβ-CdomB; and the C-domain of FAK, GST-FAK-Cdom, and its fragment, GST-FAK-CdomB.

The CAKβ C-domain, GST-CAKβ-Cdom, was bound by GST-Hic5(fl), GST-Hic5-Ndom, and Hic-5 from WFB cells but not by GST-Hic5-Cdom (Fig. 5B). The CAKβ N-domain was not bound by these GST fusion proteins or by Hic-5 from WFB cells (data not shown). The FAK C-domain, GST-FAK-Cdom, was also bound by GST-Hic5(fl) and GST-Hic5-Ndom (data not shown). When the two regions of the divided CAKβ C-domain were tested for the binding, only the extreme C-terminal region, GST-CAKβ-CdomB, was bound by GST-Hic5(fl) and GST-Hic5-Ndom (Fig. 5, C and E). These results are consistent with the results obtained by dot blots, in which the λZIPPII phage plaques of the original cbp-1 clone induced to produce the fusion protein were probed with 32P-labeled GST-CAKβ-CdomA and GST-CAKβ-CdomB; the positive signal was obtained only with GST-CAKβ-CdomB (data not shown). The same region of FAK contained the binding site; only the extreme C-terminal region of the FAK C-domain, GST-FAK-CdomB, was bound by GST-Hic5(fl) and GST-Hic5-Ndom (Fig. 5F). It was noted that the extreme C-terminal halves of the CAKβ and FAK C-domains gave better signals than the whole C-domains in the blot overlay assays. These results indicate that a common structure in the extreme C-terminal halves of the CAKβ and FAK C-domains has specific affinity to the N-domain of Hic-5.

CAKβ Forms a Complex with Hic-5, a Focal Adhesion Protein

FIG. 4. Localization at focal adhesions of Myc-tagged Hic-5 expressed in WFB cells. pHis5-Myc plasmid for expression of Myc-tagged Hic-5 was transfected into WFB cells at 2.2 μg/5-cm dish by the use of TFX (Promega, Madison, WI). The transfected cells grown on glass coverslips coated with rat tail collagen were cultured for 2 days. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were doubly stained with anti-Myc (A) and with rhodamine-conjugated phalloidin (B) and were viewed under a fluorescence microscope. Pairs of photographs, A and B, were taken at the same height from the dish surface. Magnification was ×1361.

Coimmunoprecipitation of CAKβ with Hic-5 from WFB Cell Lysate—When Hic-5 was immunoprecipitated with anti-Hic-5 from the lysate of WFB cells, CAKβ was coimmunoprecipitated with Hic-5 (Fig. 7, lanes 4 and 10). This association of CAKβ with Hic-5 was found when the lysate was prepared in a lysis buffer containing 1% Nonidet P-40 as the detergent. The addition of sodium deoxycholate at 0.5% to the lysis buffer prevented successful demonstration of the coimmunoprecipitation. As shown in Fig. 2, the anti-paxillin monoclonal antibody immunoprecipitated Hic-5 (Fig. 7, lane 12 of middle) in addition to paxillin, which migrated as a broad band behind Hic-5; thus, CAKβ was also found in the immunoprecipitate with this anti-paxillin.
CAKβ Forms a Complex with Hic-5, a Focal Adhesion Protein

**Fig. 6.** Specific in vitro binding of Hic-5 and paxillin from the WFB cell lysate to the GST fusion proteins of the full-length CAKβ and of the C-terminal regions of CAKβ and FAK. The binding of Hic-5 and paxillin (Pax) to the GST fusion proteins of CAKβ and its fragments, and a fragment of FAK is shown. Fifteen μg each of protein and the indicated GST fusion proteins (fusion proteins), GST-CAKβfl, GST-CAKβ-Ndom, GST-CAKβ-CdomA, GST-CAKβ-CdomB, and GST-FAK-CdomB was bound to glutathione-agarose (10 μl each of packed volume). The beads were mixed with 1.8 mg of protein of the WFB cell lysate (lysate) where indicated by plus signs, allowed to stand 4 h at 4 °C, and then washed three times with the lysis buffer. Bound proteins were separated by SDS-PAGE in a 10% gel, blotted onto a PVDF membrane, and probed with anti-Hic-5 (top), anti-paxillin (anti-Pax middle), and anti-CAKβ (bottom) followed by visualization of positive bands by ECL. In the anti-Hic-5 blot of the proteins bound to GST-FAK-CdomB (top, lane 12), Hic-5 was separated into upper and lower bands due to the migration of GST-FAK-CdomB at the position between these two bands. In lane 13, Hic-5 immunoprecipitated from the WFB cell lysate with anti-Hic-5 was run as a reference. blot, immunoblot.

(Fig. 7, lane 12 of top). Hic-5 was resolved by SDS-PAGE into double bands just above immunoglobulin heavy chains. When the association of Hic-5 and CAKβ was examined in the reverse direction by immunoprecipitating CAKβ from the WFB cell lysate, it was hard to find Hic-5 in the anti-CAKβ immunoprecipitate by blotting with anti-Hic-5 (Fig. 6, lane 2 and 3 of middle). In an attempt to show immunoprecipitation of Hic-5 with CAKβ, we immunoprecipitated CAKβ with different anti-CAKβ antibodies: anti-CAKβ(C-a), anti-CAKβ(N), anti-CAKβ(N)-mAb, and anti-CAKβ(C)-mAb. In the immunoprecipitates from the WFB cell lysate with any of these anti-CAKβ antibodies, no significant amount of Hic-5 was demonstrated by blotting with anti-Hic-5 (Fig. 7). However, as shown in Fig. 11, immunoprecipitation with CAKβ of a tyrosine-phosphorylated protein migrating at the position of Hic-5 was found by blotting with anti-phosphotyrosine. Moreover, when the A-431 cell lysate was used, a small amount of Hic-5 was found by blotting with anti-Hic-5 in the immunoprecipitates with anti-CAKβ(C-a) and anti-CAKβ(C)-mAb (data not shown). A small amount of paxillin, but no Hic-5, was found immunoprecipitated with FAK from the WFB cell lysate (Fig. 7, lane 8) (the blotting with anti-paxillin is not shown). Paxillin was faintly detected in the immunoprecipitates with anti-CAKβ(N), anti-CAKβ(N)-mAb, and anti-CAKβ(C)-mAb by extended blotting with anti-paxillin (data not shown). As shown below, immunoprecipitation of Hic-5 with CAKβ was clearly demonstrated in COS-7 cells expressing these proteins from transfected cDNA constructs.

**Inhibition of CAKβ Coimmunoprecipitation with Hic-5 by GST Fusion Proteins of the Extreme C-terminal Portions of CAKβ and FAK**—The association of the C-terminal region of CAKβ with Hic-5, shown in Fig. 5 by blot overlay assay and in Fig. 6 by pull-down assay, was confirmed by showing an inhibition of CAKβ immunoprecipitation with Hic-5 from the WFB lysate. The addition of the extreme C-terminal region of CAKβ fused to GST, GST-CAKβ-CdomB, to the WFB cell lysate prior to the immunoprecipitation with anti-Hic-5 markedly interfered with the CAKβ immunoprecipitation with Hic-5 (Fig. 7, lane 5 of top). This inhibition of the CAKβ association with Hic-5 was also found when the corresponding C-terminal region of FAK, GST-FAK-CdomB, was added to the lysate, but the inhibition was not found when the N-domain of CAKβ, GST-CAKβ-Ndom, was added (Fig. 7, lanes 6 and 7 of top). These results are consistent with the in vitro binding data shown in Figs. 5 and 6.

Analysis of the Association of CAKβ and Hic-5 by the Use of Deletion Mutants of CAKβ Expressed in COS-7 Cells from Transfected cDNA Constructs—COS-7 cells endogenously express a small amount of Hic-5 but almost no CAKβ. In the experiments shown in Fig. 8, CAKβ and Hic-5 were expressed in COS-7 cells from transfected cDNA constructs. In this analysis of the CAKβ association with Hic-5 by immunoprecipitation with anti-Hic-5 and anti-CAKβ from the lysates of
transfected COS-7 cells, we were able to show the coimmunoprecipitation of CAKβ with Hic-5 and Hic-5 with CAKβ (Fig. 8, lanes 7 and 8). In these experiments, CAKβ was expressed as C-terminally HSV-tagged CAKβ and Hic-5 was expressed as N-terminally Myc-tagged Hic-5. There may be at least two reasons for this successful demonstration of Hic-5 coimmunoprecipitation with CAKβ. We found that the anti-Myc monoclonal antibody detected Hic-5 at a sensitivity significantly higher than that with anti-Hic-5 (the data of immunoblotting with anti-Hic-5 are not shown in Fig. 8). It was also noted that Hic-5 with an N-terminal Myc-tag was expressed at a significantly high level in COS-7 cells.

To determine the region of CAKβ responsible for the intracellular association with Hic-5, three deletion mutants of CAKβ were coexpressed with Hic-5 in COS-7 cells and examined for coimmunoprecipitation with Hic-5. Immunoprecipitation with anti-Hic-5 and with anti-CAKβ gave the same results for the association of Myc-tagged Hic-5 and the CAKβ mutants (Fig. 8). The association was not affected in deletion mutants dl 741–903 (lanes 11 and 14). No association with Hic-5 was found in deletion mutant dl 741–903 of CAKβ (Fig. 8, lanes 9 and 10). The results indicated that a region of the CAKβ C-terminal domain was responsible for the intracellular association with Hic-5.

**Tyrosine Phosphorylation of Hic-5**—The results shown above indicated that Hic-5 was a protein highly related to paxillin in its structure and function. It is known that paxillin is markedly tyrosine-phosphorylated upon activation of FAK (41). Therefore, we examined whether Hic-5 was tyrosine-phosphorylated upon activation of CAKβ and in Src-transformed cells. Hic-5 was strongly tyrosine-phosphorylated in 3Y1 and WFB cells treated with pervanadate (Fig. 9). Mobility retardation was observed in Hic-5 when the protein was heavily phosphorylated. Hic-5 in Src-transformed 3Y1 cells, SR-3Y1, was also significantly tyrosine-phosphorylated as compared with the protein in 3Y1 cells (Fig. 9). In accordance with the data reported by Shibanuma et al. (23, 24), SR-3Y1 cells, a transformed cell line, contained much smaller amounts of Hic-5 than the untransformed counterpart, 3Y1 cells (Fig. 9A, lanes 1 and 3).

When CAKβ in serum-starved WFB cells was activated by stimulation with serum, lysophosphatidic acid, or endothelin or by exposure to hypertonic osmotic stress, the levels of Hic-5 tyrosine phosphorylation were enhanced parallel with the levels of CAKβ tyrosine phosphorylation (Fig. 10). These results indicated that Hic-5 was tyrosine-phosphorylated in Src-transformed cells and also when CAKβ was activated.

**CAKβ and Hic-5 Were Tyrosine-phosphorylated in Parallel in WFB Cells either Exposed to Hypertonic Osmotic Stress or Stimulated with Lysophosphatidic Acid**—The association of Hic-5 with CAKβ was examined under conditions where the tyrosine phosphorylation of CAKβ was enhanced. The level of CAKβ tyrosine phosphorylation decreased upon detachment of WFB cells from culture dishes by trypsinization (Fig. 11, bottom, lane 3 as compared with lane 5). The tyrosine phosphorylation of CAKβ was enhanced by stimulation of WFB cells with lysophosphatidic acid (Fig. 11, lane 7) and by exposing the cells to hypertonic osmotic stress (Fig. 11, lane 9). The amounts of CAKβ coimmunoprecipitated with Hic-5 did not significantly change under these various conditions of WFB cells where the levels of CAKβ tyrosine phosphorylation varied (Fig. 11).

However, blotting with anti-phosphotyrosine revealed that the tyrosine-phosphorylated CAKβ present in anti-Hic-5 immunoprecipitates decreased upon detachment of WFB cells from culture dishes by trypsinization (Fig. 11, lane 4) and increased upon stimulation of the cells with lysophosphatidic acid and exposure of the cells to osmotic stress (Fig. 11, lanes 8 and 10). A tyrosine-phosphorylated band was found above CAKβ in the anti-Hic-5 immunoprecipitates; this band was most prominent when cells were stimulated with lysophosphatidic acid (Fig. 11, lane 8) but was also found in cells adhering on dishes and when cells were exposed to osmotic stress (Fig. 11, lanes 6 and 10).
FIG. 10. Stimuli that activate CAKβ enhance tyrosine-phosphorylation of Hic-5 in WFB cells. Confluent WFB cells in 10-cm dishes were kept for 10 h in Iscove’s medium without serum (lane 1, serum-starved). The cells were stimulated with 10% serum for 10 min (lane 2), 2 μM lysophosphatidic acid (LPA) for 3 min (lane 3), or 0.2 μM endothelin (endothelin 1, Sigma) for 5 min (lane 5) or stimulated by osmotic stress (lane 4) via exposure of the cells to a medium containing 0.3 M sorbitol for 5 min. CAKβ and Hic-5 were immunoprecipitated from 1.5 mg of protein of each of the cell lysates prepared from these cells. The cell lysates were prepared in a lysis buffer containing 0.5% sodium deoxycholate and 0.1% SDS in addition to 1% Nonidet P-40. After blotting onto PVDF membranes, the separated proteins were probed with anti-Hic-5, anti-CAKβ(C-a) (anti-CAKβ) or anti-phosphotyrosine (anti-PTyr) as indicated. Anti-CAKβ(C-a) was used as an anti-CAKβ. Faintly stained heavy chains of rabbit immunoglobulins (Ig) overlapped at the bottom of the Hic-5 band. i.p., immunoprecipitation; blot, immunoblotting.

another experiment (data not shown), this band above CAKβ revealed with anti-phosphotyrosine was resolved into double bands, an upper major band and a lower minor band. The former band above CAKβ was far stronger than expected from the intensity with anti-phosphotyrosine blotting of Hic-5 coimmunoprecipitated with CAKβ from cells exposed to osmotic stress, whereas it was not possible to make the former band visible by blotting with anti-phosphotyrosine (Fig. 11, lane 2). Careful examination indicated that Hic-5 immunoprecipitated with anti-Hic-5 from cells on dishes and from cells stimulated with lysophosphatidic acid was also stained faintly with anti-phosphotyrosine.

FIG. 11. CAKβ and Hic-5 were simultaneously tyrosine-phosphorylated in WFB cells either exposed to hypertonic osmotic stress or stimulated with lysophosphatidic acid. Sixteen 10-cm dishes of confluent and quiescent WFB cells of the same culture lot were prepared and divided into four groups. Cells on the dishes of the first group were stimulated in Iscove’s medium with 2 μM lysophosphatidic acid (LPA) for 10 min at 37 °C (lanes 7 and 8). Cells on the dishes of the second group were exposed to hypertonic osmotic stress (Osm) for 20 min at 37 °C by replacing the culture medium with 2 ml of Iscove’s medium containing 0.3 M sorbitol (lanes 9 and 10). Cells on the dishes of the third group were trypsinized (off) after washing with ATLV solution (20); the cells were detached from dishes and allowed to stand at 37 °C for 20 min after the addition of trypsin inhibitor (lanes 3 and 4). Cells on the dishes of the fourth group were directly submitted to analysis without treatment (on) (lanes 5 and 6). Cell lysates were prepared from these four groups of cells. Hic-5, CAKβ, FAK, and paxillin (Pax) were immunoprecipitated from 1.2 mg of protein of these WFB cell lysates with anti-Hic-5 and anti-CAKβ(C-a) (anti-CAKβ) bound to protein A-Sepharose and anti-FAK and anti-paxillin monoclonal antibodies bound to anti-mouse IgG-agarose as indicated at i.p. As controls, normal rabbit Ig and normal mouse IgG1 bound to these beads were used for immunoprecipitation from the cell lysate (lanes 1 and 2). In lane 2, anti-CAKβ(C-a) itself bound to protein A-Sepharose was run as a control. FAK and paxillin were immunoprecipitated from the lysate of on cells. The immunoprecipitates were subjected to SDS-PAGE in a 7.5% gel, and the separated proteins were blotted onto a PVDF membrane. The membrane was cut at the 89-kDa prestained marker protein into high and low molecular weight regions. The proteins in the low molecular weight region were first probed with anti-Hic-5 to obtain the data shown at the top. The proteins in the low molecular weight region were first probed with anti-Hic-5 to obtain the data shown at the top. The proteins in both regions were reprobed and then reprobed with anti-phosphotyrosine, 4G10, to obtain the data shown at the bottom; the heavy chains of the mouse immunoglobulins (Ig) used in the immunoprecipitation were stained at the position below Hic-5 in lanes 11, 12 and 13 of the bottom. Binding of antibody probes was visualized either by alkaline phosphatase (bottom) or by peroxidase (ECL) (top). Pax, paxillin; i.p., immunoprecipitation; blot, immunoblotting.

A Small Portion of CAKβ Is Present in WFB Cells at the Site of Focal Adhesions—It was shown that the localization of FAK at focal adhesions is dependent on the association of FAK with paxillin (18), which is targeted to focal adhesions by itself (38). Since CAKβ bound Hic-5 and paxillin and a fraction of CAKβ coimmunoprecipitated with Hic-5 from the WFB cell lysate, we examined WFB cells by immunofluorescence with CAKβ and FAK to determine the site of focal adhesions. The major portion of CAKβ in WFB cells was found in the perinuclear region and in the cytoplasm (Fig. 12A). In the same cell line, FAK localized at focal adhesions (Fig. 3a). At the cell periphery of well spread WFB cells, where focal adhesions were seen by staining with anti-paxillin and anti-vinculin, CAKβ was faintly immunostained at focal adhesions (Fig. 12). Thus, a small amount of CAKβ was found at the sites of focal adhesions in WFB cells. However, CAKβ was immuno-
stained diffusely at the focal adhesions, not in the well-confined, rodlike patchy structure of focal adhesions, an image obtained by immunostaining vinculin, paxillin, and Hic-5. When WFB cells were doubly stained for Hic-5 and vinculin, the localization of Hic-5 at the cell periphery overlapped with that of vinculin (data not shown).

Analysis of Tyrosine Phosphorylation Levels of CAKβ and Hic-5 in WFB Cells: Effects of Trypsinization and Replating the Cells onto Dishes Coated with Fibronectin or Poly-L-lysine—The changes in the tyrosine phosphorylation levels of FAK, CAKβ, and Hic-5 were compared by trypsinization of WFB cells and replating the cells on dishes coated with either fibronectin or poly-L-lysine. The tyrosine phosphorylation levels of FAK decreased upon detachment of well spread WFB cells from culture dishes by trypsinization, and the levels were recovered by incubating the cells at 37 °C for 45 and 90 min after plating them onto dishes coated with fibronectin (data not shown). Plating the cells on poly-L-lysine dishes was not effective to recover the tyrosine phosphorylation level of FAK. These results on FAK obtained in WFB cells are consistent with those reported on FAK in BALB3T3 cells and NIH3T3 cells (11, 27). In parallel experiments, it was found that the tyrosine phosphorylation levels of CAKβ in WFB cells decreased on trypsinization and recovered on incubation at 37 °C for 45 min after plating the cells, but this recovery was observed on plating the cells not only onto fibronectin dishes but also onto poly-L-lysine dishes, although the effect of fibronectin was somewhat stronger than the effect of poly-L-lysine (data not shown). Moreover, a longer incubation of the cells for 90 min after replating did not enhance the tyrosine phosphorylation levels of CAKβ.

The tyrosine phosphorylation levels of Hic-5 were also examined in the same WFB cells trypsinized and replated as described above. Hic-5 was tyrosine-phosphorylated only to a limited degree in well spread WFB cells, and the levels of tyrosine phosphorylation did not consistently change on trypsinization and replating of the cells (data not shown).

DISCUSSION

We identified Hic-5 in a search for CAKβ-binding proteins by cDNA expression cloning. Hic-5 had an amino acid sequence highly similar to paxillin. The results shown in this paper demonstrated that Hic-5 was a component of focal adhesions. This finding disagrees with the original identification by Shibamura et al. (23, 24) that Hic-5 is localized in nuclei. A binding of the Hic-5 LIM domain to certain DNA sequences was postulated (24). We consider that their assumption that Hic-5 is a nuclear protein was drawn from weak evidence obtained by the use of a less specific anti-Hic-5 antibody (23), although a possible function of Hic-5 in the nucleus cannot be excluded at present. In this paper, we have confirmed the Hic-5 localization at focal adhesions by immunostaining with anti-Myc in WFB cells expressing the Myc-tagged Hic-5 from a cDNA construct.

The cell biological functions of Hic-5 should be reevaluated from the standpoint that Hic-5 is a component of focal adhesions. Recently, Brown et al. (38) showed that LIM domains are responsible for targeting paxillin to focal adhesion. They identified the LIM3 domain as the principal determinant of paxillin localization at focal adhesions. The four contiguous LIM domains of Hic-5 are most similar to those of paxillin among the known LIM domains (42); the highest similarity is found in each corresponding domain. Among the corresponding four LIM domains of Hic-5 and paxillin, the LIM3 domains of these proteins are most highly conserved with an identity of 70.6% in the amino acid sequences. In addition to a novel role of the LIM domain as protein dimerization motifs (36), it has been proposed that the LIM domain recognizes a tyrosine-containing tight turn (37). The identity of the protein recognized by the LIM3 domains of paxillin and Hic-5 is most interesting because Hic-5 may also be targeted to focal adhesions through its LIM3 domain.

We found that the Hic-5 N-domain directly associated in vitro with the C-terminal regions of CAKβ and FAK (Figs. 5 and 6). Moreover, the association of Hic-5 with CAKβ was shown in the lysates of WFB cells and of those COS-7 cells that were doubly transfected with CAKβ and Hic-5 cDNA constructs. Brown et al. (38) localized on paxillin the binding sites of FAK and vinculin to small stretches of amino acid sequences, which they named LD motifs. They pointed out high sequence similarity between paxillin and Hic-5 in the LD motifs. The paxillin-binding sites on FAK and vinculin have also been identified by Tachibana et al. (18) and named paxillin-binding subdomains 1 and 2 (PBS1 and PBS2). These subdomains are also found in CAKβ at amino acid residues 875–891 (PBS1) and 990–998 (PBS2). The PBS1 and PBS2 of CAKβ and CAKβ are highly conserved, being 58.8% identical in PBS1 and 77.8% identical in PBS2. The Hic-5 N-domain bound to the CAKβ C-domain and its fragment containing both PBS1 and PBS2 (Figs. 5 and 6).

The presence of Hic-5 in association with CAKβ in the WFB and COS-7 cell lysates (Figs. 7, 8, and 11) suggested that Hic-5 was a component in signaling pathways downstream of CAKβ. The results shown in Figs. 7 and 8 are consistent with the prediction that the CAKβ amino acid residues containing both PBS1 and PBS2 are essential for the binding of CAKβ to Hic-5. In the deletion mutant of CAKβ, dl 741–903, PBS2 was present, but PBS1 was deleted. In addition to the association of Hic-5 with CAKβ, the association of paxillin with CAKβ and that of Hic-5 with FAK were also found. It is possible that there are differences in affinities of the associations between FAK/CAKβ and paxillin/Hic-5. In this relation, we noted that the GST fusion protein of the full-length CAKβ, GST-CAKβ(N), had much less affinity to paxillin than to Hic-5 (Fig. 6, lane 4). Moreover, the results in Fig. 6 suggest less binding of Hic-5 to GST-FAK-CdomB than to GST-CAKβ-CdomB.

A high sequence similarity was found between the first LD motif of paxillin and the Hic-5 amino acid sequence translated from the “5′-untranslated region,” which was presumed on...
mouse Hic-5 cDNA by Shibanuma et al. (23), of human Hic-5 cDNA, cbp-1 (Fig. 1). The published mouse Hic-5 amino acid sequence (23) contains only three LD motifs lacking the one corresponding to the first LD motif of paxillin. The translational initiation site of the Hic-5 cDNA presumed by Shibanuma et al. (23) does not fit to the sequence context for translational initiation (39) and may not be the true translational initiation site of the Hic-5 mRNA. The level of Hic-5 protein expression in the cDNA transfected cells has not been shown by these authors (23, 24). The expression of Hic-5 from constructs of the original human Hic-5 cDNA, cbp-1, was quite low in the transfected cells (data not shown). We will continue our efforts to identify the N-terminal methionine and to sequence further 5′-upstream region of Hic-5 cDNA by isolating other Hic-5 cDNA clones.

Although Hic-5 is almost exclusively localized at focal adhesions in WFB cells (Fig. 3) and CAKβ has specific affinity to Hic-5 and paxillin, only a limited, small fraction of CAKβ localized at focal adhesions in WFB cells (Fig. 12). This subcellular localization of CAKβ was different from that of FAK, a large portion of which was found at focal adhesions in WFB cells (Fig. 3). The major portion of CAKβ in WFB cells is present in the perinuclear region and cytoplasm (Fig. 12). In cultured epithelial cells, CAKβ was also found at the cell-to-cell borders in addition to the perinuclear regions and cytoplasm. A small amount of CAKβ present at focal adhesions in WFB cells was found only by careful examination and was not immunostained in images of typical components of focal adhesions such as paxillin, vinculin, Hic-5, and FAK (Fig. 12). This intracellular localization of CAKβ was compatible with our finding that CAKβ is present in association with microvilli, cilia, and axons in rat tissue images (16). Tachibana et al. (18) showed that FAK localizes at focal adhesions through its binding to paxillin. It was shown that the C-domain of CAKβ exclusively localized at focal adhesions when this domain was singly expressed in chicken embryo fibroblasts from a designed recombinant cDNA construct (43). It is obvious that CAKβ has an intrinsic property of localizing at focal adhesions via its C-domain. In most cells, however, the major portion of CAKβ does not localize at focal adhesions. The reason for this subcellular localization of CAKβ at sites other than focal adhesions is a focus of our current study. The regulatory mechanism of CAKβ localization might be important in understanding the unique functions of CAKβ different from FAK. We (1) previously showed that the tyrosine phosphorylation of CAKβ is not enhanced in response to plating rat 3Y1 fibroblasts onto fibronectin. Thereafter, different results have been reported on the changes of the CAKβ tyrosine phosphorylation in response to integrin signaling (14, 44, 45). The subcellular localization of CAKβ shown in Fig. 12 may explain our finding that the tyrosine-phosphorylation was not regulated by cell-to-substratum adhesion at focal adhesions.

Although the amino acid sequences of Hic-5 and paxillin are similar at the LIM domains and also at the LD motifs, the other portions of the Hic-5 N-domain are not similar to those of the paxillin N-domain. Paxillin has a role as a signal-integrating protein or a docking protein. It was shown that the Src family protein kinases associate with paxillin by their SH3 domains (46). It was also shown that the transforming protein v-Crk binds with its SH2 domain to the phosphorylated sequences at tyrosine residues 31 and 118 of the paxillin N-domain (47–49). Although Hic-5 does not share these ligand sequences, the proline-rich sequence found at amino acid residues 14–20 and some tyrosine-phosphorylated sequences of Hic-5 may also have ligand specificities to some SH3 and SH2 domains. A tyrosine residue at position 43 of Hic-5, which is not found in paxillin, is in a sequence context indicative of a possible phosphorylation site. The Hic-5 coimmunoprecipitated with CAKβ from the lysate of WFB cells stimulated by osmotic stress was selectively tyrosine-phosphorylated as compared with the total cellular Hic-5 (Fig. 11). This result indicates functional coupling of CAKβ and Hic-5. However, it remains unanswered which tyrosine kinase, CAKβ, Src, or another one, is responsible for the phosphorylation of Hic-5. Thus, the portion of the Hic-5 N-domain outside the LD motif is probably the site important in signal transduction. It seems important to study whether the cell biological effects of Hic-5 reported by Shibanuma et al. (24) are related to unique downstream signals, which are possibly generated from Hic-5 but not from paxillin. Because Hic-5 and paxillin have several properties in common, related but different roles are expected for their functions.

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