Phosphorylation of Hic-5 at tyrosine 60 by CAKβ and Fyn

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Received 2 February 2000; received in revised form 5 May 2000

Edited by Veli-Pekka Lehto

Abstract Hic-5 is a CAKβ-binding protein localized at focal adhesions. Here we show that overexpression of CAKβ or Fyn, but not FAK, enhanced the tyrosine phosphorylation of coexpressed Hic-5 in COS-7 cells. These phosphorylations were further augmented by stimulating cells with osmotic stress. The Y60F mutant of Hic-5 was not phosphorylated, and Hic-5 phosphorylated on tyrosine 60 was bound specifically to the SH2 domain of Csk. Coexpression experiments revealed that the phosphorylation of Hic-5 by CAKβ required the kinase activation of CAKβ and binding of Hic-5 by CAKβ. Specific phosphorylation of Hic-5 by CAKβ and Fyn may activate a signaling pathway mediated by Hic-5.

Key words: Cell adhesion kinase β; Fyn; Hic-5; Tyrosine phosphorylation; Osmotic stress

1. Introduction

We identified Hic-5 by expression cloning as a CAKβ-binding protein [1]. Hic-5 cDNA was initially identified by Shibanuma et al. [2] as one of the cDNA clones derived from mRNA induced by hydrogen peroxide. Hic-5 localizes at focal adhesions in fibroblasts [1]. In a rat fibroblast line WFB, which expresses CAKβ and Hic-5 endogenously, Hic-5 and CAKβ are coimmunoprecipitated and Hic-5 is tyrosine phosphorylated in parallel with CAKβ when cells were stimulated with osmotic stress [1]. Hic-5 is related to paxillin in structure and these two proteins share four LD motifs and four LIM domains [1]. One of the LD motifs mediates the association of Hic-5 with CAKβ, which, also called PYK2 [3], RAFTK [4], CadTK [5], is a non-receptor protein tyrosine kinase of the focal adhesion kinase (FAK) family. LIM domains determine the localization of paxillin and Hic-5 at focal adhesions [6–9]. Other proteins also associate to the LD motifs and the LIM domains [10–12]. Similarity in amino acid sequence between Hic-5 and paxillin is not high outside the LD and LIM regions and these two proteins probably have distinct functions. There is no amino acid sequence in Hic-5 corresponding to the ligand sequences of paxillin where Crk and Src bind with their SH2 or SH3 domain. Recently Hic-5 was shown to be a coactivator of nuclear receptors [13], suggesting that Hic-5 has functions both in the cytoplasm and in the nucleus. In this paper, we showed that Hic-5 was phosphorylated at tyrosine 60 by CAKβ and also by Fyn. The phosphorylation by CAKβ required the kinase activity of CAKβ, tyrosine residue 402, an Src-binding site of CAKβ, and the Hic-5-binding sites of CAKβ. This phosphorylation on tyrosine 60 created in Hic-5 a possible binding site for the SH2 domain of Csk.

2. Materials and methods

2.1. Recombinant cDNAs

All the CAKβ cDNA constructs used here originated from clone 17N, a full-length rat CAKβ cDNA [14]. Construction of the deletion mutant of paxillin-binding subdomain 1 (dl741–903) was described previously [1]. To construct CAKβ(Y402F) and CAKβ(K457A), site-directed mutations were introduced by using oligonucleotides GAATCTCTGCAAAGATATCTGACTCTAT and CTTACAGGTCGCGACGGCCA. The former changed the coding sequence of tyrosine 402 to phenylalanine and the latter changed lysine 457 to alanine. The construction of CAKβ(CGM), in which proline residues 717 and 859 were changed to alanine, was previously described [15]. These CAKβ cDNA constructs were cut with *Bst*UI and *Eco*RI and subcloned into pcDNA3. CAKβ fused to glutathione S-transferase (GST), GST-CAKβ, was expressed from pGEX-CAKβ, which was constructed by subcloning a *Bam*HI/*Eco*RI fragment of pEBG-CAKβ [1] into pGEX-2T. GST-CAKβ(Y402F) was constructed by swapping the *Nsi*I fragments, which cover the mutation site, between pcDNA3-CAKβ(Y402F) and pGEX-CAKβ. For construction of the expression vector of FAK, a *Bam*HI site was introduced juxtaposed to the translation initiation codon of a cDNA encoding full-length wild-type mouse FAK [16], by using PCR primers CGGATCCATGGCAGCTGCTTATCTTGACC and CGCATATGACTGAGGCGAAATCCATAG. The FAK cDNA was then inserted into the *Bam*HI site of pcDNA3Myc [1].

cDNA constructs for the expression of Hic-5 were derived from the original human Hic-5 cDNA clone, cbp-1 [1], to which ATG was added at its 5′ end. This ATG was assigned as the first methionine codon of Hic-5 according to Thomas et al. [8]. Addition of the first methionine codon to cbp-1 was done by PCR using the forward primer GTCTAGACAGACCGTGCATCATGGAGGACCTGGATGCCCTG, which was designed to introduce the *Xba*I site at the 5′ terminus. The construct was cut with *Xba*I and *Eco*RI and subcloned into *Xba*I/*Eco*RI-digested pcDNA3.1(−) to make pcDNA3-Hic-5. Y60F mutation of Hic-5 was introduced by site-directed mutagenesis using an oligonucleotide, CATACCGTGCTGAACAGGTGGTCC. Oligonucleotide site-directed mutagenesis was done by the Kunkel method [17] by the use of a Mutan-K mutagenesis kit (Takara). The mutation and fidelity of DNA amplification by PCR were confirmed by DNA sequencing. Fyn expression vector ME18S-Fyn was a kind gift of T. Yamamoto [18]. Vectors expressing GST fusion proteins of Csk-SH2 [19], Crk-I-SH2 [20], and GST-Fyn-SH2 and GST-Src-SH2 [21] were generously provided by M. Okada, M. Matsuda, and S. Kobayashi, respectively.

2.2. Antibodies

Preparation of rabbit serum against CAKβ residues 670–716 (anti-CAKβ(C-a), designated anti-CAKβ(C) in this paper) and rabbit serum against the CAKβ N-terminal region, anti-CAKβ(N), were as described [1]. A mixture of anti-CAKβ(N) and anti-CAKβ(C) was used for Western blotting. Production of the affinity purified rabbit

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Abbreviations: CAKβ, cell adhesion kinase β; FAK, focal adhesion kinase; SH, Src homology; Csk, C-terminal Src kinase; GST, glutathione S-transferase

PII: S0014-5793(00)01597-0

anti-Hic-5 antibody, anti-Hic-5, was described previously [1]. Anti-GST was derived from rabbit serum against GST-Hic-5, from which the anti-GST fraction was isolated by using GST coupled to CNBr-activated Sepharose beads. Monoclonal antibodies against phosphotyrosine (4G10, mouse IgG2) and paxillin (clone 349, mouse IgG1) were purchased from Upstate Biotechnology, Inc. and Transduction Laboratories, respectively. The Fyn polyclonal antibody against the N-terminal region of Fyn was previously described [22]. Fyn monoclonal antibody (Fyn(15)) was a product of Santa Cruz Biotechnology, Inc. Anti-phosphotyrosine (PT66) conjugated agarose beads were purchased from Sigma.

2.3. Expression of cDNA constructs in COS-7 cells, and cell stimulation with osmotic stress

COS-7 cells were grown in a culture medium (Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin and 50 μg/ml streptomycin). COS-7 cells growing close to confluence in 10 cm culture dishes were transfected with up to 10 μg of expression plasmid constructs as specified in the legends using DEAE-dextran and used 48 to 70 h after the transfection. Where indicated, cells were stimulated with osmotic stress by exposing them to 0.3 M sorbitol in culture medium at 37°C for 8 min before cell lysis.

2.4. Recombinant adenovirus

The *Xba*I-*Eco*RI fragment of pcDNA3-Hic-5 containing the entire coding sequence was blunted and inserted into the *Swa*I site of the adenovirus cosmid cassette, pAxCAwt [23]. A recombinant adenovirus for expression of Hic-5, AxCASHic5, was obtained by transfecting 293 cells with the above cosmid together with adenoviral DNA cut with *Eco*T22I, according to the COS-TPC method [24]. For infection with AxCASHic5, the medium was removed from rat 3Y1 cells growing at 70% confluence in 10 cm dishes and the cells were infected with AxCASHic5 in 1 ml of culture medium at a moi of 10. The cells were supplied with 10 ml of culture medium 1 h after infection, and used 2 days later.

2.5. Immunoprecipitation and immunoblotting

Cells were lysed in RIPA buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% NP40, 25 mM HEPES pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.3 mg/ml benzamidine, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mg/ml soybean tripsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1.5 mM Na_3VO_4). This RIPA buffer dissociates binding between Hic-5 and CAKβ [1]. Cell lysates were incubated with the antiserum specified and with 10 μl of protein A-Sepharose beads for 4 h or more up to overnight. The antisera used for the immunoprecipitation of CAKβ and Hic-5 were anti-CAKβ(N) and anti-Hic-5, respectively. After three washes with the buffer used in the lysis of cells, bound proteins were eluted with sample buffer for SDS–polyacrylamide gel electrophoresis (PAGE) at 90°C for 5 min, and analyzed by SDS–PAGE in 10% gels. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). The membranes were blocked with 3% bovine serum albumin (BSA) in TBST (25 mM Tris, pH 7.6, 136 mM NaCl, 0.1% Tween-20) at 60°C for 20 min and incubated at room temperature for 2 h in TBST containing the specified antibody and 1% BSA. The membranes were then washed three times in TBST, incubated at room temperature for 1 h in TBST containing goat anti-mouse (Kirkegaard and Perry Laboratories, MD, USA) or goat anti-rabbit (Sigma) antibodies conjugated with horseradish peroxidase, and finally washed three times in TBST. The peroxidase conjugated antibodies were then located by chemiluminescence (ECL, Amersham Corp.).

3. Results

3.1. Tyrosine phosphorylation of Hic-5 by Fyn and CAKβ

We previously showed that the tyrosine phosphorylation of Hic-5 was enhanced in parallel with that of CAKβ when rat WFB fibroblasts, which express these proteins endogenously, were serum-starved and then stimulated with lysophosphatidic acid or exposed to high-osmotic stress to activate CAKβ [1]. COS-7 cells were doubly transfected with two different expression vectors, one encoding Hic-5 and the other coding one of the non-receptor tyrosine kinases; Fyn, FAK, or CAKβ. Co-expression of CAKβ, but not of FAK, with Hic-5 resulted in the tyrosine phosphorylation of Hic-5 (Fig. 1A, lanes 5 and 7). In agreement with our previous observation in WFB cells, the phosphorylation by CAKβ was further enhanced by treating the cells with high-osmotic stress (lane 8). Interestingly, Fyn also increased the phosphorylation of Hic-5 (lane 3). Moreover, this phosphorylation was also enhanced by the

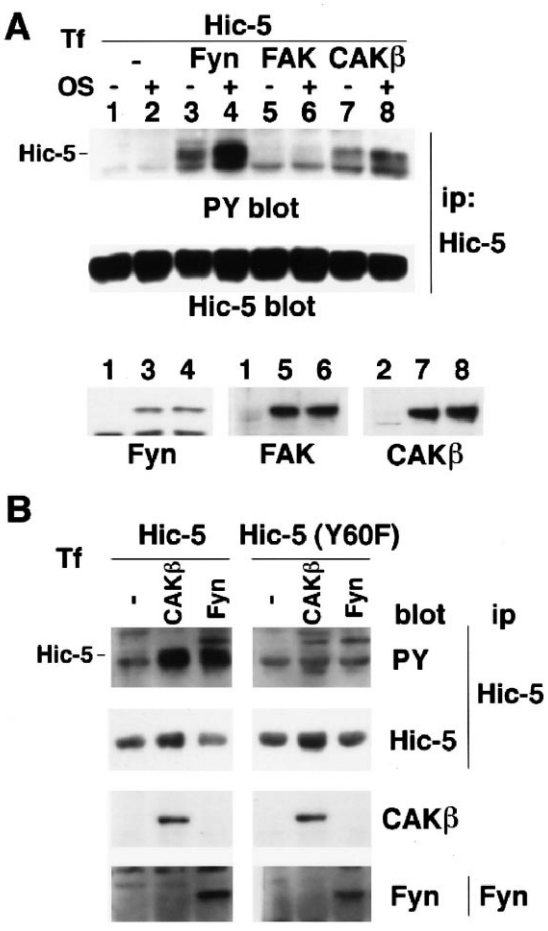

Fig. 1. Phosphorylation of Hic-5 on tyrosine 60 by CAKβ and Fyn. A: COS-7 cells were transiently transfected with pcDNA-Hic-5 (Hic-5), together with an empty expression plasmid (−, lanes 1, 2) or an expression plasmid of Fyn (lanes 3, 4), FAK (lanes 5, 6) or CAKβ (lanes 7, 8) as indicated. After 2 days of culture, cells were lysed directly (OS −) or after they were stimulated by osmotic stress (OS +). Aliquots of the cell lysates were taken aside to monitor the expression of introduced kinases; the expression of Fyn was determined after immunoprecipitation and CAKβ and FAK were determined without immunoprecipitation. The remaining extracts were subjected to immunoprecipitation with anti-Hic-5. Immunoprecipitates and the total cell extracts were analyzed by immunoblotting with anti-phosphotyrosine (PY), anti-Fyn, anti-FAK, and anti-CAKβ antibodies. Hic-5 immunoprecipitates were probed with anti-PY and then probed again with anti-Hic-5 after the bound antibody was stripped off. B: COS-7 cells were doubly transfected with two different expression plasmids, one for expression of Hic-5, either the wild-type (wt) or Y60F mutant (Y60F), and the other, either an empty expression plasmid (−) or an expression plasmid of CAKβ or Fyn as indicated. After 2 days of culture, the cells were stimulated with osmotic stress and then lysed. Lysates were analyzed by immunoblotting as described in A.

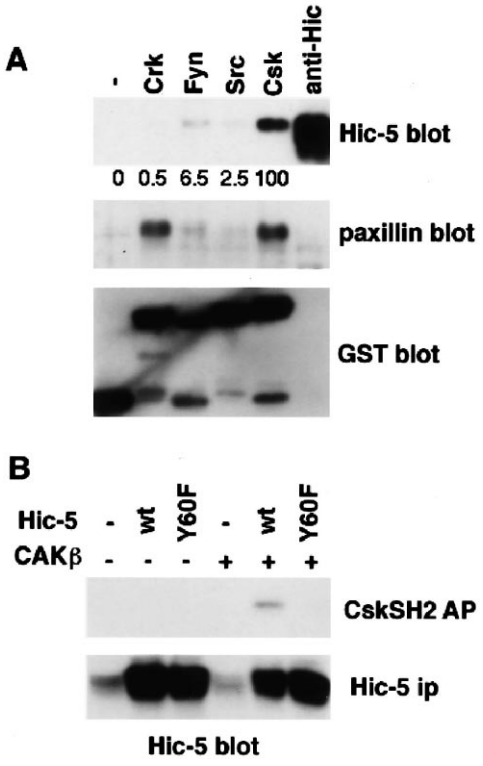

Fig. 2. Phosphorylation of Hic-5 on tyrosine 60, which on phosphorylation was bound with specificity to Csk-SH2. A: 3Y1 cells infected with a recombinant adenovirus encoding Hic-5 were treated with 0.5 mM pervanadate for 10 min and then the cells were lysed. Cell lysate was incubated at 4°C for 4 h with GST (−), GST-Crk-SH2 (Crk), GST-Fyn-SH2 (Fyn), GST-Src-SH2 (Src), and GST-Csk-SH2 (Csk) along with glutathione-Sepharose beads. As a control, the same amount of the lysate was immunoprecipitated with anti-Hic-5. Relative intensities of the signals were given under the photo. Bound proteins were analyzed by immunoblotting with anti-Hic-5 and with anti-GST antibodies. The Hic-5 blot was probed again with anti-paxillin after the bound antibody was stripped off. B: COS-7 cells were transfected with two different plasmids, one encoding Hic-5, either wild-type (wt) or Y60F mutant (Y60F), or an empty vector (−), and the other encoding CAKβ (+) or an empty vector. After cell stimulation by osmotic stress, lysates were prepared and subjected first to affinity precipitation (AP) with GST-Csk-SH2. Then, Hic-5 was immunoprecipitated (ip) from the supernatant obtained on the affinity precipitation assay. Proteins bound to GST-Csk-SH2 and anti-Hic-5 were analyzed by immunoblotting with anti-Hic-5 antibody.

osmotic stress (lane 4). The percentages of Hic-5 phosphorylated by CAKβ and Fyn were determined by comparing the amount of Hic-5 precipitated by anti-phosphotyrosine antibody with the total amount of Hic-5. In a rough estimation on Hic-5 from cells exposed to osmotic stress, 4% and 16% of Hic-5 present in a cell were found to be tyrosine phosphorylated when CAKβ and Fyn, respectively, were coexpressed. When these kinases were not coexpressed, the tyrosine phosphorylation of Hic-5 was not detectable.

3.2. Phosphorylation of Hic-5 at tyrosine 60

Although the structure of Hic-5 is similar to that of paxillin in the alignment of domains and in the amino acid sequences of LIM domains and LD motifs, there are no sequences on Hic-5 corresponding to two major phosphorylation sites known in paxillin, tyrosine residues 31 and 118 [25,26]. There is, however, one tyrosine at residue 60 in the N-terminal region of Hic-5, which has preceding aspartic acid residues and is likely to be phosphorylated by protein tyrosine kinases. We prepared a Hic-5 mutant (Y60F) in which this tyrosine residue was changed to phenylalanine. COS-7 cells were doubly transfected with two different plasmid vectors, one encoding either CAKβ or Fyn and the other encoding either wild-type Hic-5 or the Y60F mutant of Hic-5. The wild-type Hic-5 was found to be tyrosine phosphorylated when coexpressed with CAKβ or Fyn, but Y60F Hic-5 was hardly phosphorylated under the same conditions (Fig. 1B), indicating that tyrosine 60 was the major site of Hic-5 phosphorylation.

3.3. Specific binding of the SH2 domain of Csk to phosphorylated Hic-5

One of the major consequences of tyrosine phosphorylation of signaling proteins is the formation of a specific binding site to the SH2 domain of a ligand molecule. We examined GST fusion proteins of the SH2 domains from Crk, Fyn, Src and Csk, to determine their ability to bind the tyrosine phosphorylated Hic-5. Rat 3Y1 cells were infected with a Hic-5-expressing recombinant adenovirus, treated with pervanadate and then lysed to use in the affinity-binding experiment. We found that the phosphorylated Hic-5 present in the 3Y1 cell lysate was bound specifically to the Csk SH2 domain (Fig. 2A, top panel). The amount of Hic-5 bound to the Csk SH2 was 200 times higher than the amount bound to the Crk SH2. In contrast to Hic-5, paxillin endogenously present in the pervanate-treated cells was bound to the SH2 domains of both Crk and Csk (Fig. 2A, middle panel). To prove that the phosphorylation of tyrosine 60 creates the site for this association with the Csk SH2 domain, lysates of COS-7 cells overexpressing both CAKβ and Hic-5, either wild-type or the Y60F mutant, were subjected to affinity precipitation with the GST-Csk SH2 domain. As expected, wild-type Hic-5 was pulled down with the GST-Csk SH2 domain and Hic-5(Y60F) was not (Fig.

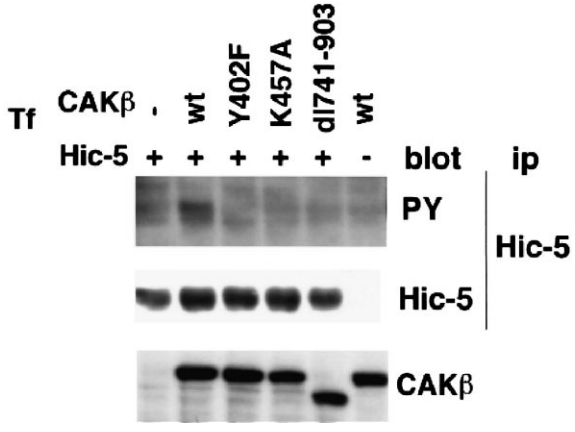

Fig. 3. Phosphorylation of Hic-5 by CAKβ mutants. COS-7 cells were transfected with two different expression plasmids, one encoding Hic-5 (+) or an empty vector (−), and the other encoding CAKβ (wt), CAKβ(Y402F), CAKβ(K457A), or CAKβ(dl741–903), or an empty vector. Three days after the transfection, cells were stimulated by osmotic stress and lysed. An aliquot of the lysates was used to monitor the expression of CAKβ. The other lysate was subjected to immunoprecipitation with anti-Hic-5. The cell lysates and the immunoprecipitates were analyzed by immunoblotting with anti-CAKβ and anti-phosphotyrosine antibodies, respectively. Hic-5 immunoprecipitates were then probed with anti-Hic-5 after the bound anti-PY antibody was stripped off.

2B). In the control cells where CAKβ was not expressed, Hic-5 was not pulled down with the GST-Csk SH2 domain. The cell biological importance of this Hic-5-Csk association is still not clear because we were not able to show coimmunoprecipitation of Csk with Hic-5 in lysates from cells and tissues. However, the results shown in Fig. 2 indicate that the Hic-5 phosphorylated at tyrosine 60 binds to certain SH2 domain(s) with high specificity.

3.4. Phosphorylation of Hic-5 by mutant forms of CAKβ

We tried to answer the question whether the phosphorylation of Hic-5 required direct binding of Hic-5 to CAKβ. COS-7 cells were doubly transfected with the vector encoding Hic-5 along with the vector encoding wild-type or mutant CAKβ. The cells were stimulated by osmotic stress and lysed, and then the phosphorylation of Hic-5 was assayed by Western blotting. As shown in Fig. 3, Hic-5 was not phosphorylated when coexpressed with CAKβ(K457A), CAKβ(Y402F), or CAKβ(dl741–903); these are a kinase-minus mutant, an Src-binding site mutant [27], and a deletion mutant of a paxillin-binding site, which was previously shown to be defective in the association with Hic-5 [1], respectively. Importantly, CAKβ(Y402F) is not functional as a kinase on Hic-5. The data suggest that the activation of CAKβ kinase and direct association of CAKβ with Hic-5 were both necessary for the phosphorylation of Hic-5. On the other hand, we were not able to detect by Western blot analysis any coprecipitation of Hic-5 with Fyn and of Fyn with Hic-5 in overexpression system of COS-7 cells (data not shown).

4. Discussion

We previously showed that in WFB cells, which express CAKβ and Hic-5 endogenously, the level of Hic-5 tyrosine phosphorylation was increased when cells were exposed to high-osmotic stress which activates CAKβ [1]. COS-7 cells do not endogenously express Hic-5 at a detectable level. When exogenously expressed alone, Hic-5 was not tyrosine phosphorylated even after stimulation by osmotic stress, although COS-7 cells endogenously express c-Src and FAK. The tyrosine phosphorylation of Hic-5 expressed in COS-7 cells was markedly enhanced by coexpression of Fyn or CAKβ, but not by coexpression of FAK. These results indicated the presence of an intracellular signaling pathway triggered by mechanical stress on a cell, leading to the phosphorylation of Hic-5 by Fyn and/or CAKβ. Although we have already shown that CAKβ binds directly to Hic-5 [1], there is no report so far describing an osmosensor that couples to the CAKβ activation. Recently, Fyn, but not Src, was shown to increase tyrosine phosphorylation of cortactin in CHO cells upon osmotic stress [28]. If some Src family kinase is activated in COS-7 cells at the downstream of mechanical stress, CAKβ can possibly be activated by the Src family kinase. Activated Src family kinase also helps to maintain the activated state of CAKβ by binding to CAKβ at the phosphorylated tyrosine 402 [29,30] and thus protecting the phosphoryl residue from the attack of phosphatases. On the other hand, transient expression of exogenous Hic-5 and Fyn in COS-7 cells also resulted in the phosphorylation of Hic-5 in the absence of CAKβ expression. This result indicated that Fyn could phosphorylate Hic-5 without a help of CAKβ. However, we were not able to show an association of Fyn with Hic-5 in the coexpressed cells. It is known that Hic-5 does not have a binding site for Src-SH3 which is present in paxillin. Therefore, it cannot be excluded as a possibility that overexpressed Fyn phosphorylated Hic-5 non-specifically. However, because Hic-5 was phosphorylated more effectively in Fyn expressing cells as compared with cells expressing CAKβ, there could be some functional connection between Fyn and Hic-5. Fyn, though not binding directly to Hic-5, may associate with Hic-5 possibly through endogenous CAKβ which did exist in COS-7 cells in barely detectable level, or through some adapter protein(s) yet remains to be characterized.

Many of the downstream signaling pathways well known to be activated by FAK are also set in motion once CAKβ is activated. However, the upstream signaling pathways linked to CAKβ activation are still largely unknown. Although CAKβ is activated, like FAK, following stimulation of receptors coupled to phospholipase C activation, only a few upstream pathways unique to CAKβ have been reported. These are the activation of CAKβ in the downstream of Fyn, JAK3, and Syk following stimulation of immunoreceptors and cytokine receptors [31–33]. Osmotic stress most effectively stimulates CAKβ in many cultured cells. Although FAK is also found to associate with Hic-5, either FAK overexpression or stimulation of FAK by ligating integrin, or by osmotic stress does not increase Hic-5 phosphorylation ([34] and data not shown). Phosphorylation of Hic-5 may thus be specific to CAKβ activation. Although Hic-5 localizes at focal adhesions in fibroblasts [1], CAKβ is generally found diffusely distributed throughout the cytoplasm [1,35,36]. The intracellular site of the Hic-5 phosphorylation is thus unclear. However, the C-terminal non-catalytic domain of CAKβ expressed as a fragment was found to localize exclusively at focal adhesions [35], indicating a possible function of CAKβ at focal adhesions on some special, controlled occasions. It is also possible that CAKβ phosphorylates Hic-5 in some other compartments of the cell, such as the nucleus.

Tyrosine phosphorylation of signaling proteins often generates a specific binding site to a modular protein domain mediating protein assembly, such as SH2 and PTB domains. Our data indicate a possibility that Csk binds the phosphorylated Hic-5 with its SH2 domain. This implies that a negative feedback loop of CAKβ activation is formed: Csk can inactivate Src family kinases associated with CAKβ, leading to the dissociation of Src family kinases from CAKβ and then to the exposure of the phosphorylated tyrosine 402 to phosphatases, such as PTP-PEST [10]. However, we have not so far succeeded in proving an in vivo association of Csk with Hic-5. Indeed, the amount of Hic-5 pulled down with GST-Csk SH2 was not very high. Although weaker binding does not necessarily mean that the association is not functioning within cells, the results may imply possible presence of another protein(s) that endogenously interact with the tyrosine phosphorylation site of Hic-5. However, a demonstration of a specific interaction of tyrosine phosphorylated Hic-5 with some SH2 domains, in this case Csk SH2, is by itself important because the intracellular signaling through tyrosine phosphorylation relies on specific protein–protein interactions. Indeed, the binding between the Csk SH2 and tyrosine phosphorylated Hic-5 was shown to be specific, suggesting a function of this phosphorylated site in some intracellular signaling pathway. Proteins other than Csk that bind to Hic-5 at the site of phosphorylated tyrosine 60 are now in search to be found.

Acknowledgements: We are grateful to Dr. Tadashi Yamamoto, Dr. Steve K. Hanks, Dr. Masato Okada, Dr. Michiyuki Matsuda, and Dr. Shin Kobayashi for providing us with the Fyn expression vector, FAK cDNA, GST-Csk(SH2), GST-Crk(SH2), and GST-Fyn(SH2)/GST-Src(SH2), respectively. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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