Interaction of two proline-rich sequences of cell adhesion kinase β with SH3 domains of p130^{Cas}-related proteins and a GTPase-activating protein, Graf

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Cell adhesion kinase β (CAK β) is a protein tyrosine kinase closely related to focal adhesion kinase (FAK) in structure. CAK β contains two proline-rich sequences within its C-terminal region. Since proline-rich sequences present in the corresponding region of FAK are known to mediate protein–protein interactions by binding to SH3 domains, we investigated binding of CAK β to a panel of SH3 domains. Affinity precipitation from rat brain lysate revealed selective interactions of CAK β with glutathione S-transferase (GST)-fused SH3 domains of p130^{Cas}(Cas)-related proteins and Graf. Mutational analysis indicated that the proline-rich sequences of CAK β mediate this interaction. Each

of the two proline-rich sequences fused to GST bound directly to these SH3 domains in dot blot analysis. A competitive binding assay revealed that the first proline-rich sequence of CAK β preferentially associated with the SH3 domain of Cas. The second proline-rich sequence of CAK β bound to the SH3 domain of Graf with higher specificity than the corresponding proline-rich sequence of FAK. Finally, we showed co-immunoprecipitation of CAK β with Graf from rat brain lysate. These results indicate that CAK β associates *in vivo* with Graf through its SH3 domain.

INTRODUCTION

Protein–protein interactions regulated through such modular binding domains as SH2 and SH3 play pivotal roles in intracellular signal transduction [1,2]. The Src homology 3 (SH3) domain, a well-conserved region comprised of about 60 amino acids, has been shown to mediate protein–protein interactions in various events in cell biology as, for instance, signalling via receptor protein tyrosine kinase (PTK), subcellular localization of proteins [1,2], cytoskeletal organization [3], initiation of endocytosis [4], activation of the NADPH oxidase system [5], and HIV growth [6]. A series of SH3 ligand sites identified so far is rich in proline residues and contains the consensus PXXP motif in the binding core [1]. Three-dimensional studies of SH3s complexed with ligands indicated that these ligands formed a left-handed polyproline type II (PPII) helix and fell into two general classes [7], class I and class II, which pseudo-symmetrically fit the binding pocket in mutually opposite orientations [8,9]. Although the overall structure of SH3 domains determined to date is very similar, their amino acid sequence similarity is rather restricted, suggesting that variable residues in the SH3 domain may allow variety in ligand specificity.

Cell adhesion kinase β (CAK β), also known as PYK2/RAFTK [10–12], is a non-receptor-type PTK structurally related to focal adhesion kinase (FAK). These PTKs exhibit a unique domain structure, with a centrally located catalytic domain flanked by large N- and C-terminal regions, which are 418 and 330 amino acid residues in CAK β , respectively. These non-catalytic regions of CAK β , as well as those of FAK, serve as multiple protein binding sites, indicating a docking protein-like function of these PTKs. Ligand proteins common to these PTKs include Fyn [13,14], c-Src [13,15,16], paxillin [17,18], p130^{Cas} (Cas) [19–21], HEF1 [22,23] and Grb2 [11,15]. On the other hand, only FAK is reported to bind the p85 subunit of phosphatidylinositol 3-

kinase (PI3K) [24], Efs [19], and Graf [25]. Recently, we demonstrated binding of CAK β to Hic-5, a paxillin-related protein [26]. Although the interactions listed above are not necessarily specific to either CAK β or FAK, restricted homology and, therefore, a difference between the amino acid sequences of the N- and C-terminal regions of the two PTKs may indicate selective ligand affinities that result in distinct functions.

The C-terminal region of CAK β has two proline-rich sequences that contain the PXXP motif and are potential binding sites for SH3 domains. The first proline-rich sequence, P⁷¹³PPKPSRP, and the second one, P⁸⁵⁵PQKPPRL, in CAK β have sequences similar to the corresponding regions of FAK, A⁷¹¹PPKPSRP and P⁸⁷⁴PKKPPRP, respectively. Although these proline-rich sequences in FAK have been reported to serve as SH3 ligands [19,20,24,25], so far there has been no direct evidence that those in CAK β mediate any interactions with SH3 domains.

In this paper, we tested a panel of SH3 domains for binding to CAK β . We showed that CAK β selectively interacted with Graf, as well as Cas, through SH3-mediated bindings. Although Graf was originally cloned as a FAK-binding protein with Rho/Cdc42 GTPase-activator activity [25], our data showed that the binding of the SH3 domain of Graf to the second proline-rich sequence of CAK β was more selective than that to the corresponding sequence of FAK. Co-immunoprecipitation of CAK β and Graf from brain homogenate suggested that these two proteins interact *in vivo* through SH3-mediated binding.

EXPERIMENTAL

GST-fusion proteins and their radiolabelling

Fragments of cDNAs covering amino acid sequences designated in this paper CasSH3, HEF1SH3, EfsSH3 and EfsPR, which correspond to residues 1–67 of mouse Cas, residues 1–67 of

Abbreviations used: PTK, protein tyrosine kinase; CAK β , cell adhesion kinase β ; FAK, focal adhesion kinase; GST, glutathione S-transferase; PI3K, phosphatidylinositol 3-kinase; PLC γ 2, phospholipase γ 2; GAP, GTPase-activating protein; LPA, lysophosphatidic acid; RB, rat brain.

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human HEF1, residues 1–70 and 295–353 of mouse Efs, respectively, were prepared by polymerase chain reaction (PCR) from cDNA clones of mouse Cas (pBS-Cas) [19], kindly provided by Dr. S. K. Hanks, HEF1 (ATCC 103620), and mouse Efs [27]. Plasmids for expression of GST fusion proteins were constructed as follows. Each pair of oligonucleotide primers used in the PCR was tagged with *Bam*HI and *Eco*RI sites to facilitate properly orientated, in-frame cloning of these cDNA fragments into pGEX-2T, -2TK or -3X expression vectors (Pharmacia). Every DNA fragment prepared by PCR was sequenced by the dideoxy sequencing method using Sequenase (US Biochemical) in order to check for possible misreadings by Taq polymerase. Vectors for expression in bacteria of GST-GrafSH3 [25], and GST-Crk(N)SH3 and -Crk(C)SH3 [28] were kindly provided by Dr. J. T. Parsons and by Dr. M. Matsuda, respectively. *Escherichia coli* strain BL21(DE3) was transformed with the pGEX recombinant plasmids. The GST-fusion proteins were induced in *E. coli* and purified as described [27]. Preparations of GST-CAK β -Ndom, GST-CAK β -Cdom, GST-CAK β -CdomA, GST-CAK β -CdomB, GST-FAK-CdomA, GST-FAK-CdomB, GST-SrcSH3, GST-FynSH3, GST-LckSH3 and GST-PLC γ 2SH3 were described elsewhere [26,29]. Radiolabelled probes were prepared as described previously [26]. Briefly, 0.1 nmol of each purified GST-fusion protein was radiolabelled by incubation with 100 μ Ci of [γ - 32 P]ATP and 100 units of the catalytic subunit of cAMP-dependent protein kinase (Sigma) for 30 min at room temperature.

Antibodies and antiserum

The anti-Graf rabbit antiserum used in this study was prepared by immunization of New Zealand White male rabbits with GST-GrafSH3. This antiserum was used either without purification or after affinity-purification on a column of immobilized immunogen following removal of anti-GST. Affinity-purified rabbit polyclonal anti-CAK β (C-a), anti-CAK β (N) and anti-GST antibodies, and the anti-CAK β mouse IgG₁ monoclonal antibody, anti-CAK β (N)-mAb, were prepared as described [26]. Monoclonal anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology. Rabbit polyclonal anti-FAK (IgG fraction of antiserum), monoclonal anti-rabbit immunoglobulin (clone RG-16) conjugated with alkaline phosphatase or peroxidase, goat anti-mouse IgG (Fc-specific) conjugated with alkaline phosphatase, and rabbit IgG were purchased from Sigma.

Western blotting

Whole rat brain (1.0 g) was homogenized in 10 ml of lysis buffer (10 mM Tris/HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1 % Nonidet P-40, 1 mM sodium vanadate, 100 units/ml aprotinin, 10 mM NaF, 20 μ g/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride), and the supernatant was obtained by centrifugation at 15000 *g* for 20 min at 4 °C. COS-7 (ATCC CRL 1651), PC-12 (ATCC CRL 1721) and WFB [30], a rat fibroblast line, cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10 % heat-inactivated (56 °C for 30 min) fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 μ g/ml streptomycin. Cells grown to confluence in 10-cm dishes were harvested and lysed on ice in the lysis buffer. Before stimulation of cells, the medium in confluent monolayer cultures of the cells in 10-cm dishes was removed and replaced 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 0.5 to 60 min with 2 μ M dioleoyl L- α -lysophosphatidic acid (LPA) (Sigma), exposed to hypertonic osmotic stress at 37 °C for 0.5 to 60 min by replacing the medium with 2 ml of warm Iscove's

medium containing 0.3 M sorbitol, or treated for 15 min with 1 mM pervanadate [42]. For affinity precipitation, 1.0 mg of protein of the rat brain lysate or 0.5 mg of protein of lysate from COS-7 cells transfected with CAK β constructs was incubated for 6 h with 5.0 μ g of a GST-SH3 fusion protein immobilized on 10 μ l of glutathione-agarose beads. For immunoprecipitation, 1.0 mg or 5.0 mg of protein of the rat brain lysate was incubated for 6 h with the anti-Graf antiserum or anti-CAK β (N) antibody bound to Protein A-Sepharose beads. The beads were then washed three times with lysis buffer. Bound proteins were eluted by boiling in SDS/PAGE sample buffer, separated by SDS/PAGE in a 10 % gel, and blotted to a PVDF membrane (Millipore). The membrane filters were blocked with 3 % (w/v) BSA in TBST (25 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.05 % Tween 20) for 20 min at 60 °C and then incubated with primary antibodies in TBST containing 1 % BSA for 2 h at room temperature. The membrane were washed five times with TBST and incubated with secondary antibodies conjugated with peroxidase or alkaline phosphatase in TBST for 1 h, followed by five washes in TBST. Positive bands were detected either by the ECL system (Amersham) or by incubation in nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphatase (BCIP) (Sigma).

Mutagenesis and transient expression of CAK β in COS-7 cells

cDNA constructs expressing mutants of CAK β , CAK β -P⁷¹⁷A, CAK β -P⁸⁵⁹A and CAK β -P^{717/859}A, were prepared as follows. First, wild-type CAK β was cloned into pcDNA3 vector (Invitrogen). Site-directed mutations which substitute Ala for targeted Pro were introduced by the Kunkel method (TaKaRa) with synthesized nucleotide primers 5'-GCCGGCTGGCCTTG-GGTGG-3' and 5'-GCCGTGGTGCCTTCTGGGG-3'. The primers were designed to introduce new sites for restriction enzymes so that generation of proper mutations was confirmed by digestion with the restriction enzymes. These vectors were transfected into confluent COS-7 cells at 5 μ g/10-cm dish using the DEAE-dextran method [31]. After 3 days, the cells were harvested and used for further analysis.

Dot blot analysis and competitive binding assay

GST or GST fusion proteins (0.3 pmol) were dotted onto nitrocellulose filters (Schleicher & Schuell). After being dried, the filters were blocked for 1 h at 4 °C with binding buffer (20 mM Hepes pH 7.5, 75 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05 % Nonidet P40) containing 5 % (w/v) skim milk. Filters were rinsed, and incubated in sealed bags for 16 h at 4 °C in 10 ml of binding buffer containing 2.0×10^6 c.p.m. of a radiolabelled GST fusion protein as a probe. For the competitive binding assay, filters dotted with GST or GST fusion protein were prepared as described above and incubated by rotation in microtubes for 16 h at 4 °C in 1.0 ml of binding buffer containing a 3.0 nM 32 P-labelled GST-CasSH3 probe and different amounts of unlabelled GST-SH3 proteins added as competitors of the probe. Filters were then washed three times with binding buffer, and bound radioactivities were made visible and quantified using a Fujix BAS-2000 bio-imaging system (Fujifilm Photo Film Co., Ltd) after 30 min exposure [32].

RESULTS

Selective binding of CAK β to GST-SH3 fusion proteins

Since CAK β has two proline-rich sequences containing the core consensus motif, PXXP, for SH3 binding [7], we first examined

Figure 1 Selective binding of CAK β to GST-SH3 fusion proteins

Rat brain (RB) lysate (1.0 mg of protein) was incubated with GST or the indicated GST-SH3 fusion protein bound to glutathione-agarose beads. Associated proteins were separated by SDS/PAGE, blotted onto a PVDF membrane, and probed with anti-CAK β (N)-mAb (**A**) or anti-GST antibody (**B**). Lane 1 where 100 μ g of protein of total RB lysate was applied shows immunoreactive CAK β present in the lysate. The positions of CAK β (arrow), GST (arrow), GST-SH3 domains (bracket), and molecular-mass markers (numbers in kDa) are shown.

Figure 2 Binding of wild-type and mutant forms of CAK β expressed in COS-7 cells to GST-CasSH3 and to GST-GrafSH3

About 0.5 mg of protein of lysates prepared from COS-7 cells transfected with cDNA constructs expressing CAK β and CAK β mutants as indicated was subjected to immuno- or affinity-precipitation (AP/IP) by the use of immobilized anti-CAK β (N) polyclonal antibody, GST, or GST-SH3 domains. Bound proteins were separated by SDS/PAGE, blotted onto PVDF membranes, and probed with an anti-CAK β (N)-mAb (**A**) or anti-GST antibody (**B**). The positions of CAK β , GST, GST-SH3 domains, and molecular-mass markers (in kDa) are indicated.

the binding of CAK β to a panel of SH3 domains. Immobilized GST fusion proteins that contained the SH3 domains of c-Src, Fyn, Lck, PLC γ 2, Crk(N), Crk(C), Cas, HEF1, Efs and Graf were incubated with rat brain lysate. Anti-CAK β immunoblotting revealed that only the SH3 domains of Cas and Graf associated strongly with CAK β (Figure 1). SH3 domains of Cas-related proteins HEF1 and Efs bound CAK β very weakly. Other GST-SH3s tested and GST, a control, did not bind CAK β at a detectable level. The origin of and reason for the immunostaining of a $\sim$ 70 kDa band which was detected by the anti-CAK β antibody in the lanes of proteins bound to the Src family SH3 domains, is not known. The results clearly demonstrated that CAK β selectively interacted with the SH3 domains of Cas and Graf. Similar results were reported for binding to the proline-rich regions of FAK [20,25]. However, the SH3 domains of HEF1 and Efs affinity-precipitated more FAK than CAK β from WFB cell lysate (results not shown).

B

Probes	Binding of probes to the SH3 indicated					
	Cas	HEF1	Efs	Graf	Fyn	GST
CAK β -Ndom	-	-	-	-	-	-
CAK β -Cdom	+++	+++	++	+++	-	-
CAK β -CdomA	+++	+++	++	+	-	-
CAK β -CdomB	+	+	+	+++	-	-
FAK-CdomA	+++	+++	++	++	-	-
FAK-CdomB	++	++	+	+++	-	-
Efs-PR	-	-	-	-	+++	-

Figure 3 Binding of different regions of CAK β fused to GST to various GST-SH3 domains

(**A**) The regions of CAK β used as probes are indicated by arrows. Locations on CAK β of the kinase domain (Kinase), YAEI sequence (YAEI), the first proline-rich sequence (PPPKP⁷¹⁷SRP), and the second proline-rich sequence (PPQKP⁸⁵⁹PR) are schematically shown with numbered positions of amino acid residues. (**B**) GST or GST-SH3 proteins dot-blotted on nitrocellulose membranes were incubated with radiolabelled probes of the indicated GST fusion proteins. After the membranes were washed, binding of GST-CAK β fusion proteins was determined as described. The relative binding intensity was graded as strong (+++, >30% of the intensity obtained in the binding of GST-EfsPR to GST-FynSH3), middle (++, 5–30%), weak (+, 0.5–5%) and not detectable (–, <0.5%).

Two proline-rich sequences mediate association of CAK β with SH3 domains of Cas and Graf

We next investigated whether the association of CAK β with these SH3 domains was mediated through proline-rich sequence(s). Three mutants of CAK β , CAK β -P⁷¹⁷A, CAK β -P⁸⁵⁹A and CAK β -P^{717/859}A, were generated. They contained either or both of the two mutations that disrupt PXXP motifs: Pro⁷¹⁷ to Ala in the first proline-rich sequence and Pro⁸⁵⁹ to Ala in the second proline-rich sequence. These wild-type and mutant CAK β s were expressed in COS-7 cells and subjected to affinity precipitation. As shown in Figure 2, GST-CasSH3 and GST-GrafSH3 both affinity-precipitated a detectable amount of wild-type CAK β (lanes 2, 3), CAK β -P⁷¹⁷A (lanes 10, 11) and CAK β -P⁸⁵⁹A (lanes 14, 15), but CAK β -P^{717/859}A (lanes 6, 7) was not affinity-precipitated by these SH3 domains. These data indicated that CAK β interacted with the SH3 domains of Cas and Graf through its proline-rich sequences and that each of the two proline-rich sequences could participate in this association.

Binding specificity of the proline-rich sequences in CAK β and FAK

To investigate the binding specificity of individual proline-rich sequences of CAK β and FAK, ³²P-labelled GST fusion proteins containing each of these sequences (Figure 3A) were tested for their binding to GST-SH3 proteins dot-blotted onto nitrocellulose membranes. CAK β -CdomA and CAK β -CdomB, which contain the first and the second proline-rich sequences of CAK β , respectively, as well as FAK-CdomA and FAK-CdomB, which contain the corresponding regions of FAK, were bound by the SH3 domains of Cas, HEF1, Efs and Graf (Figure 3B). Efs-PR, which contains two proline-rich sequences known to be bound by the SH3 domains of Src family kinases [27], was not bound by these SH3 domains. The first proline-rich sequence of CAK β was preferentially bound by the SH3 domains of Cas, HEF1 and Efs, whereas the second proline-rich sequence was bound only by the

Figure 4 Competitive inhibition by various GST-SH3 domains of GST-CasSH3 binding to the regions of the CAK β C-domain fused to GST

Affinity-purified GST fusion proteins of the indicated region of the CAK β - or FAK-C-domain were dot-blotted on nitrocellulose filters and incubated with radiolabelled GST-CasSH3, as a probe, in the absence and presence of the indicated unlabelled GST-SH3 domains, added as binding competitors. After washing, the radioactivity of bound probe was determined as described. To facilitate comparison, the radioactivity of the bound probe obtained in the absence of a competitor was arbitrarily set at 10^3 for each panel.

Graf-SH3 domain with strong affinity. A similar tendency was observed for the probes derived from FAK.

In the competitive binding assay shown in Figure 4, binding of radiolabelled GST-CasSH3 to GST fusion proteins containing proline-rich ligand sequences, which were blotted on nitrocellulose membranes, was determined in the presence of unlabelled GST-SH3 proteins added as binding competitors. The effectiveness of unlabelled GST-SH3 proteins in competing for the binding of the radiolabelled probe reflects their relative affinities [34] to the ligand proteins immobilized on the nitrocellulose membrane. As shown in Figure 4, the binding of GST-CAK β -CdomA with the GST-CasSH3 probe was strongly inhibited by the addition of GST-CasSH3 itself, and reduced to some extent by the addition of GST-HEF1SH3, GST-EfsSH3 and GST-GrafSH3 (Figure 4a). These results indicated that Cas-SH3 had high relative affinity for CAK β -CdomA. On the other hand, Graf-SH3 had high relative affinity to CAK β -CdomB, because GST-GrafSH3 most strongly inhibited the binding of GST-CasSH3 to GST-CAK β -CdomB (Figure 4b). The results indicated that the affinity of Graf-SH3 to CAK β -CdomB was higher than that of Cas-SH3. Moreover, the results of binding to FAK-CdomB indicated that the affinity of Graf-SH3 to this region was lower than that of Cas-SH3 (Figure 4d). The affinity of HEF1-SH3 to CAK β -CdomA was intermediate between Graf-SH3 and Cas-SH3 (Figure 4a). The affinity of HEF1-SH3 to FAK-C domains was comparable to that of Cas-SH3. GST-EfsSH3 was not a good competitor for Cas-SH3 in any of the four binding assays.

Co-immunoprecipitation of CAK β and Graf from rat brain lysate

Graf has Rho-GAP activity and was found as a protein possibly associating with FAK [25], although no *in vivo* association of these two proteins has been shown. Nor has the complete

Figure 5 Coimmunoprecipitation of CAK β and Graf

For immunoprecipitation, 1.0 mg (lane 2 of each panel) or 5.0 mg each (lanes 3 and 4 of each panel, and lane 5 of **A**) of total soluble protein of rat brain lysate was incubated with anti-Graf, anti-CAK β (N), anti-FAK, or rabbit IgG as indicated. The proteins in the precipitates were subjected to SDS/PAGE, blotted on PVDF membranes, and immunostained with anti-Graf antiserum (**A**), a mixture of anti-CAK β (C-a) and anti-CAK β (N) polyclonal antibodies (**B**), and anti-FAK antibody (**C**). To assess the efficiency of co-immunoprecipitation, 50 μ g of protein of rat brain lysate was subjected directly to SDS/PAGE at lane 1 of each panel.

primary structure of Graf been reported so far. Our data showed that Graf was a ~ 90 kDa protein (Figure 5A). We also found that Graf was most abundant in the brain among mouse tissues examined (results not shown), which was in agreement with Northern blot analysis of Graf mRNA [25]. PC12, COS-7 and WFB cells were also found to express detectable amounts of Graf. Tyrosine-phosphorylation of Graf was enhanced in pervanadate treated cells (results not shown). When immunoprecipitation from rat brain lysate was done using anti-CAK β and anti-FAK, Graf was detected both in the anti-CAK β and anti-FAK immunoprecipitates (Figure 5A, lanes 3 and 4). CAK β was also detected in the anti-Graf immunoprecipitate (Figure 5B, lane 3), whereas FAK was not found in the anti-Graf immunoprecipitate (Figure 5C, lane 3). Neither CAK β nor Graf was detected when the precipitation was done with normal rabbit IgG. These results clearly demonstrated an *in vivo* association of CAK β with Graf.

DISCUSSION

Although CAK β and FAK are closely related in their overall structures, their subcellular and tissue distributions are different [10]. Siciliano et al. reported differential regulation of CAK β and FAK by stimulation with glutamate and depolarization in rat hippocampus [38]. However, it remains unanswered what overlapping and different roles these related kinases play in cell physiology. Recent evidence shows that the C-terminal region of CAK β mediates binding of CAK β with Grb2 [11], paxillin [18] and Hic-5 [26]. This portion of CAK β may thus act in part as a signal assembler that binds proteins involved in the upstream or downstream signalling. In this paper, we investigated the binding of proteins to the two proline-rich sequences of CAK β mediated by SH3 domains. GST fusion proteins, which were used to assess ligand candidates for modular domains mediating protein-protein interaction [33–37], were also used in several *in vitro* binding assays. We found that each of the two proline-rich sequences of CAK β was capable of interacting with the SH3 domains of Cas, HEF1, Efs and Graf, although each one was different to some degree in terms of binding specificity. This was also the case in FAK. The first and second proline-rich sequences of CAK β had high affinity to the SH3 domains of Cas and Graf, respectively. The SH3 domains of the Cas-related proteins HEF1 and Efs did not firmly associate with CAK β ,

Figure 6 The alignment of the proline-rich sequences in CAK β , FAK and other proteins

Conserved proline and basic residues at the numbered residues of the protein indicated are enclosed with boxes and ovals, respectively. In the consensus sequence, + and X represent basic and any residues, respectively. Sequences were taken from the following source: CAK β [10], FAK (GenBank M95408), PTP1B [40], PTP-PEST (GenBank M93425), dynamin I (GenBank L07807), Gab1 (Grb2-associated binder-1 [48]), Sam68 (PIR A38219), OSF (osteoclast stimulating factor, GenBank U63717), PI3K-p85 α (SWISS-PROT P27986), synaptojanin (GenBank U45479).

although their structures are closely related to the Cas SH3 domain. HEF1 has more affinity to FAK than to CAK β and this difference may be partly related to the differences in function between CAK β and FAK. Because the binding of the Efs SH3 domain was weak with all of the CAK β /FAK proline-rich sequences, Efs-SH3 may have some other unknown target protein(s), or this weak interaction itself may be significant for the CAK β /FAK function. The functional difference in signal transduction between CAK β and FAK can be at least partly explained by the differences in the binding of these proteins.

The four proline-rich sequences present in CAK β and FAK have distinct similarities in their sequence motifs (Figure 6), although their binding specificities are different. As pointed out by Feng et al. [39], amino acid residues outside the proline-rich core are important for increasing SH3-binding specificity and affinity. The consensus Arg residue at position +5 of the C-terminal side of PXXP motif found in these four sequences of CAK β and FAK indicates that they adapt the conformation of a class II ligand [8,9]. Alignment of these four sequences and the other proline-rich sequence derived from PTP1B, which is reported to bind to the SH3 domain of Cas [40], allowed us to deduce a novel consensus ligand sequence with a binding capacity to the Cas SH3 domain, PX+PX+, where + stands for Arg or Lys (Figure 6). This consensus has a basic amino acid residue at the position +2, the second X of the PXXP motif, where all of the known ligand sequences that belong to class II have a hydrophobic amino acid residue such as Leu and Val. Recently, the SH3 domain of amphiphysin was reported to have binding specificity to a similar consensus sequence [41]. By a database search, we found that amino acid sequences containing this consensus motif, PX+PX+, are present in several other proteins (Figure 6). Among them, PTP-PEST was reported to interact directly with Cas through its catalytic domain [42]. Thus, these

proteins are possible ligands to the SH3 domains of Cas, HEF1, Efs and Graf, and therefore, may possibly compete for binding to CAK β and FAK [43]. Cas, HEF1 and Efs are structurally related docking proteins. The SH3 domains of these proteins have a unique amino acid residue in their sequences, Asn¹⁰⁸ in the case of rat Cas [44], where most other SH3 domains commonly have an aromatic residue such as Tyr and Phe. Interestingly, Graf has Cys at this position. Results on the three dimensional structures of SH3 domains [7-9] indicate that these alterations in amino acid sequences would be closely related to changes in binding specificity to the characteristic basic residue present at the position +2 of the consensus ligand sequence described above; these residues on a SH3 domain and on a class II ligand can form a direct hydrophilic association, as we have previously postulated [45].

Graf is a GTPase-activating protein (GAP) of RhoA and Cdc42 [25], and is known to bind FAK *in vitro*. Interestingly, in our competition assay, it was found that the binding affinity of Graf-SH3 relative to that of Cas-SH3 was significantly high in the binding to CAK β -CdomB as compared with the binding to FAK-CdomB. In agreement with this, Graf and CAK β were coimmunoprecipitated when immunoprecipitations were done with anti-CAK β and anti-Graf, respectively, whereas coimmunoprecipitation of Graf and FAK was detected only with anti-FAK, but not with anti-Graf. Although this result could be due to a relatively lower expression of FAK in rat brain [10], it is likely that the portion of Graf in association with FAK is smaller than that in association with CAK β . It is therefore possible that Graf is more selectively associated with CAK β as compared with FAK at least in brain. In relation to this possibility, it is interesting to note that overexpression of CAK β , but not FAK, results in alteration of cell morphology in chicken embryo cells [46]; this may possibly be due to the change in RhoA/Cdc42 signalling mediated by Graf. Recently, we found that CAK β and Hic-5, a novel ligand of CAK β , were tyrosine-phosphorylated in parallel [26] when WFB cells were either stimulated with LPA or exposed to hypertonic osmotic-stress [47]. The tyrosine phosphorylation of Graf was also enhanced in WFB cells to some degree by the same treatments (results not shown). The *in vivo* interaction of CAK β and Graf shown in this paper suggests that CAK β modulates intracellular signalling pathways regulating cytoskeletal reorganization.

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