CAKβ (cell adhesion kinase β)/PYK2 (proline-rich tyrosine kinase 2) is the second protein-tyrosine kinase of the FAK (focal adhesion kinase) subfamily. It is different from FAK in that it is activated following an increase in cytoplasmic free Ca$^{2+}$. In the present study we have investigated how Ca$^{2+}$ activates CAKβ/PYK2. Calmodulin-agarose bound CAKβ/PYK2, but not FAK, in the presence of CaCl$_2$. An α-helix (F2-α2) present in the FERM (band four-point-one, ezrin, radixin, moesin homology) F2 subdomain of CAKβ/PYK2 was the binding site of Ca$^{2+}$/calmodulin; a mutant of this region, L176A/Q177A (LQ/AA) CAKβ/PYK2, bound to Ca$^{2+}$/calmodulin much less than the wild-type. CAKβ/PYK2 is known to be prominently tyrosine phosphorylated when overexpressed from cDNA. The enhanced tyrosine phosphorylation was inhibited by W7, an inhibitor of calmodulin, and by a cell-permeable Ca$^{2+}$ chelator and was almost defective in the LQ/AA-mutant CAKβ/PYK2. CAKβ/PYK2 formed a homodimer on binding of Ca$^{2+}$/calmodulin, which might then induce a conformational change of the kinase, resulting in transphosphorylation within the dimer. The dimer was formed at a free-Ca$^{2+}$ concentration of 8–12 μM and was stable at 500 nM Ca$^{2+}$, but dissociated to a monomer in a Ca$^{2+}$-free buffer. The dimer formation of CAKβ/PYK2 FERM domain was partially defective in the LQ/AA-mutant FERM domain and was blocked by W7 and by a synthetic peptide with amino acids 168–188 of CAKβ/PYK2, but not by a peptide with its LQ/AA-mutant sequence. It is known that the F2-α2 helix is found immediately adjacent to a hydrophobic pocket in the FERM F2 lobe, which locks, in the autoinhibited FAK, the C-lobe of the kinase domain. Our results indicate that Ca$^{2+}$/calmodulin binding to the FERM F2-α2 helix of CAKβ/PYK2 releases its kinase domain from autoinhibition by forming a dimer.

Key words: calmodulin, cell adhesion kinase β (CAKβ), focal adhesion kinase (FAK), FERM domain, protein-tyrosine kinase, PYK2 (proline-rich tyrosine kinase 2), transphosphorylation.

INTRODUCTION

FAK (focal adhesion kinase) and CAKβ [cell adhesion kinase β; also known as PYK2 (proline-rich tyrosine kinase 2), RAFTK (related adhesion focal tyrosine kinase) or CADTK (calcium-dependent tyrosine kinase)] constitute a group of non-receptor protein-tyrosine kinases with large homologous N- and C-terminal regions and a central kinase domain [1,2]. CAKβ/PYK2 is known to be a unique protein-tyrosine kinase activated following an increase in the cytoplasmic free-Ca$^{2+}$ concentration after stimulation of cells with ligands such as lysophosphatidic acid, endothelin, vasopressin and PDGF (platelet-derived growth factor) that bind to receptors linked to phospholipase C activation [2–4]. CAKβ/PYK2 is different from FAK in this property of Ca$^{2+}$-induced activation [2]. However, the underlying mechanism of the Ca$^{2+}$-dependent activation has remained unknown. As for the importance of the Ca$^{2+}$-induced activation of CAKβ/PYK2 in cell physiology, it has recently been shown that transient activation of CAKβ/PYK2, by a spontaneous local transient increase in the intracellular free-Ca$^{2+}$ concentration (Ca$^{2+}$ lightning) at the site of cell contacts, regulates cell–cell repulsion [5]. This result indicates that Ca$^{2+}$ signals are linked to cell–cell repulsion by CAKβ/PYK2 present in the cell periphery at the site of cell contacts via the affinity with its C-terminal region.

In the external portions of the N- and C-terminal regions of FAK and CAKβ/PYK2, there are a band FERM (band four-point-one, ezrin, radixin, moesin homology) domain (amino acid residues 37–356 in CAKβ/PYK2) [6] and a FAT (focal adhesion targeting) region (amino acid residues 877–1000 in CAKβ/PYK2), which has the structure of a four-helix bundle [7]. Between the FERM domain and the kinase domain (amino acid residues 419–679 in CAKβ/PYK2), there is a FERM-kinase linker segment containing an autophosphorylation site, Tyr$^{402}$. Between the kinase domain and the FAT region, there is a proline-rich region, which is the most divergent between FAK and CAKβ/PYK2 in terms of amino acid sequences and their lengths. Thus FERM domains occupy large portions of the N-terminal regions in CAKβ/PYK2 and FAK [6]. The FERM domain is a three-lobe domain found in many proteins such as the ERM (ezrin-radixin-moesin) family of proteins and the JAK (Janus kinase) family of tyrosine kinases [8]. It has been shown that FERM domains can mediate intermolecular interactions allowing docking with the cytoplasmic tails of transmembrane proteins. FERM domains also function in either intramolecular or homophilic intermolecular interactions. In FAK, it has been shown that the deletion of its FERM domain activated the kinase activity [9,10]. This activation was explained by release of the FAK kinase domain from the intramolecular interaction with the FAK FERM domain [11]. In CAKβ/PYK2, this intramolecular interaction between the kinase and FERM domains was not found [11], but it was shown by Dunty and Schaller [12] that the N-terminal region of CAKβ/PYK2 somehow regulated catalytic activity, subcellular localization and cell morphology. Recently, an extensive and direct contact in FAK between the FERM F2 lobe and the C-lobe of the kinase domain, including the transphosphorylation region, makes a strong interaction with the FAT region.
and the kinase C-lobe, as well as a binding of the FERM F1 lobe via the FERM-kinase linker segment to the FAK kinase N-lobe, were directly shown by the crystal structure of the autoinhibited FAK [13]. CAKβ/PYK2 exhibits greater kinase activity than FAK in vitro [12]. It has been shown that a large patch of basic residues at the third α-helix in the F2 subdomain of the FAK FERM domain is important in cell-adhesion-dependent activation of FAK and downstream signalling [11]. With the same basic residues of the FAK FERM F2 subdomain, the hepatocyte growth factor receptor Met directly interacts at its phosphorylated tyrosine residues, resulting in the activation of FAK [14]. This large patch of basic residues is also conserved in CAKβ/PYK2.

Lipinski et al. [15] showed that several substitutive mutations of CAKβ/PYK2 single amino acid residues such as I308E and Y135C, which are the mutations of the residues important in maintaining the structure of the FERM domain, markedly reduced the activation of CAKβ/PYK2 in glioma cells and reduced its capacity to stimulate glioma cell migration. Thus the results reported so far on FAK and CAKβ/PYK2 indicate that the N-terminal FERM domain is important in regulating the kinase activity of CAKβ/PYK2. However, the exact mode of this regulation remains to be clarified. In the present study, we show that CAKβ/PYK2 specifically binds Ca²⁺/calmodulin at the FERM F2 subdomain. The complex formation of CAKβ/PYK2 with Ca²⁺/calmodulin results in the activation of the protein-tyrosine kinase by forming its homodimer and stimulating transphosphorylation.

**EXPERIMENTAL**

**Materials and antibodies**

Calmodulin-agarose, PDGF-BB, vasopressin, anti-FLAG (M2) monoclonal antibody and anti-FAK (residues 1039–1052) rabbit polyclonal antibody (IgG fraction) (F-2918) were obtained from Sigma. A23187, BAPTA/AM [1,2-bis-(o-aminophenoxy)ethane-N,N,N’,N’-tetra-acetic acid tetrakis(acetoxymethyl ester)], W5, W7, W12 and W13 were purchased from Calbiochem. An anti-calmodulin monoclonal antibody, anti-phosphotyrosine monoclonal antibody (4G10) and anti-phospho-Pyk2 (Tyr402) monoclonal antibody (clone RR102) were purchased from Upstate Biotechnology. Anti-FAK (haemagglutinin; Y-11, polyclonal) and anti-(phospho-Pyk2) domain of CAKβ/PYK2 was amplified by PCR using 3'-GGATCCGGGTGTCTGAGCCCCTG-3’ and 5'-GCCTCTACCTGACCTTGGCTGACATGGTTG-3’ (antisense, 5’-GCCTCCTACCGAGCTTCGAGGCATGGTGGT-3’) were used. A vector, pcDNA3-HA1-HA4, for the expression of both N- and C-terminally HA-tagged proteins was constructed. The cDNA encoding the FERM domain of CAKβ, which corresponds to amino acid residues 37–356, was amplified by PCR with ExTaQ DNA polymerase (Takara) using oligonucleotide primers (sense, 5’-GGATCCGGGTGTCTGAGCCCCTG-3’; antisense, 5’-GCCTCCTACCGAGCTTCGAGGCATGGTGGT-3’). The PCR product was then subcloned into the pGEM-T vector (Promega). From this vector, the cDNA fragment encoding the FERM domain of CAKβ was obtained by digestion with BamHI. The BamHI fragment was subcloned into each BamHI site of the 3’×FLAG-tags vector and pcDNA3-HA1-HA4, yielding vectors expressing the FLAG-tagged and HA-tagged CAKβ FERM domains. The vectors expressing the CAKβ FERM domain with the LQ/AA mutation were constructed by the same procedures as described above except that the cDNA encoding the LQ/AA-mutant FERM domain of CAKβ was amplified by PCR using 3’×FLAG-tag vector and pcDNA3-HA1-HA4 as the template. To construct the vector expressing N-terminally Myc-tagged calmodulin, the full-length cDNA encoding human calmodulin was first amplified by PCR using a human brain cDNA library and oligonucleotide primers. The PCR product was then subcloned into the pGEM-T vector. From this vector, the cDNA fragment encoding calmodulin was obtained by digestion with BamHI and XhoI, and the fragment was subcloned into the BamHI-XhoI sites of the vector for Myc-tagged proteins described above. All of the cDNA constructs used in the present study were completely sequenced.

**Cell culture and transfections**

HeLa (ATCC CCL-2) and HEK (human embryonic kidney)-293T (ATCC CRL-11268) cells were obtained from the American Type Culture Collection. The rat fibroblast line WFB was obtained from the establisher of the line as described previously [4]. All of the cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere (95% air/5% CO₂). HeLa, HEK-293T and WFB cells were transfected with plasmids by the use of Lipofectamine Plus™ reagent (Invitrogen), HilyMax™ transfection reagent (Dojindo Laboratories) and FugeneHD™ transfection reagent (Roche) respectively.

**cDNA constructs**

The vectors expressing wild-type-, Y402F- and K457A-CAKβ/PYK2 with an N-terminal 3×FLAG-tag were constructed by inserting the BamHI fragments of the full-length wild-type-CAKβ CDNA [1], Y402F-CAKβ cDNA [16] and K457A-CAKβ cDNA [16] into the BamHI site of the vector for 3×FLAG-tagged proteins. The vector expressing N-terminally Myc-tagged FAK has been described previously [17]. The vector expressing N-terminally Myc-tagged CAKβ was constructed by inserting the cDNA fragment of the full-length CAKβ described above into the BamHI site of the vector, pcDNA3Myc, for Myc-tagged proteins [3]. The vector expressing N-terminally Myc-tagged CAKβ was prepared by PCR-based, site-directed mutagenesis of the vector expressing 3×FLAG-tagged wild-type-CAKβ/PYK2, where KOD-plus DNA polymerase (TOYOBO) and oligonucleotide primers (sense, 5’-ATGCGTGGCGCGCTGGTCTGGA-3’; antisense, 5’-GCCTCACGTACTGCTGCTGCAATGCGTT-3’). The PCR product was then subcloned into the pGEM-T vector (Promega). From this vector, the cDNA fragment encoding the FERM domain of CAKβ was obtained by digestion with BamHI. The BamHI fragment was subcloned into each BamHI site of the 3×FLAG-tag vector and pcDNA3-HA1-HA4, yielding vectors expressing the FLAG-tagged and HA-tagged CAKβ FERM domains. The vectors expressing the CAKβ FERM domain with the LQ/AA mutation were constructed by the same procedures as described above except that the cDNA encoding the LQ/AA-mutant FERM domain of CAKβ was amplified by PCR using 3×FLAG-tagged LQ/AA-mutant CAKβ/PYK2 in place of 3×FLAG-tagged wild-type CAKβ/PYK2 as the template. To construct the vector expressing N-terminally Myc-tagged calmodulin, the full-length cDNA encoding human calmodulin was first amplified by PCR using a human brain cDNA library and oligonucleotide primers. The PCR product was then subcloned into the pGEM-T vector. From this vector, the cDNA fragment encoding calmodulin was obtained by digestion with BamHI and XhoI, and the fragment was subcloned into the BamHI-XhoI sites of the vector for Myc-tagged proteins described above. All of the cDNA constructs used in the present study were completely sequenced.

**Immunoprecipitation and Western blot analysis**

Cells were washed twice with cold PBS and lysed in a lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% NP40 (Nonidet P40), a protease inhibitor cocktail (Complete™, EDTA-free; Roche), 5 mM sodium orthovanadate and 5 mM EDTA]. Cell lysates were subjected to centrifugation at 1 × 10⁵ g for 30 min at 4°C. The clarified cell lysate thus obtained was subjected to immunoprecipitation with anti-CAKβ, anti-FAK, anti-FLAG or anti-HA in the presence of protein-A-Sepharose beads (Amersham Biosciences) at 4°C for 2 h, after which the Sepharose beads were collected by centrifugation at 8000 g for 40 s at 4°C. The immunoprecipitated CAKβ and FAK were washed four times with a Lubrol buffer [50 mM Tris/HCl (pH 7.5),...
indicated primary antibody for 1 h at room temperature (20°C and 150 mM NaCl) and by incubation with the incubated for 2 h at 4°C (20 µM bovine serum albumin in TBS-T [50 mM Tris/HCl (pH 7.5) and 150 mM NaCl] and by incubation with the indicated primary antibody for 1 h at room temperature (20°C). The plots were then probed for 1 h at room temperature with a secondary antibody conjugated with alkaline phosphatase. The bands were stained with CDP-Star™ detection reagent (Amersham Biosciences). The stained images were analysed using BSA as a standard. The clarified cell lysates were washed twice with cold PBS and lysed for 10 min at 4°C in a modified lysis buffer containing either 2 mM EGTA or Ca2+/EGTA buffer was prepared according to the MaxChelator program. After centrifugation, 31% of 150 µl each or 31 fractions of 70 µl each were collected from the top to the bottom of each tube. Then 50 µl or 25 µl of a 4-fold SDS sample buffer was added to each fraction, which was then subjected to SDS/PAGE on 6% (CAKβ) or 10% (FERM domain) gels after heating at 70°C for 10 min. The separated proteins were analysed by Western blotting with anti-FLAG M2 antibody. BSA (67 kDa), rabbit muscle aldolase (tetramer, 158 kDa), bovine liver catalase (tetramer, 232 kDa) and horse spleen ferritin (24-mer, 440 kDa) were used as molecular-mass standards in the sedimentation-velocity analysis.

Affinity-binding assay with calmodulin-agarose
HEK-293T cells expressing CAKβ or one of its mutants were grown for approx. 30 h to confluence in culture dishes. The cells were washed twice with cold PBS and lysed for 10 min at 4°C in a modified lysis buffer, i.e. the lysis buffer described above but prepared without 5 mM EDTA. After centrifugation at 1 × 10^5 g for 30 min at 4°C, clarified cell lysates were obtained. Protein concentrations were determined with a Micro BCA kit (Pierce) using BSA as a standard. The clarified cell lysates (100 µg of protein in 80 µl of the modified lysis buffer) were added to a slurry (20 µl) of calmodulin-agarose beads and the suspensions were incubated for 2 h at 4°C in a modified lysis buffer containing either CaCl2, EGTA, W7 or W5, as indicated in each Figure legend. The beads with bound proteins were collected by centrifugation at 8000 g at 4°C for 40 s. The agarose beads were washed four times with a buffer of the same composition as used in each incubation. The washed beads with bound proteins were then resuspended in the SDS/PAGE sample buffer, heated at 70°C for 10 min, and subjected to analysis by SDS/PAGE and Western blotting as described above.

Determination of the free-Ca^{2+} concentration in the clarified cell lysates with and without added CaCl2
The free-Ca^{2+} concentration in the clarified cell lysates used in the incubation for the dimer formation of CAKβ/Pyk2 FERM domain was determined by the fluorescence (measured using a Hitachi 650-10S fluorescence spectrophotometer) of Fluo-4 (Molecular Probes) added at 4 µM in the lysates. The fluorescence intensity was found to be linear at the Ca^{2+} concentration of 0.6–10 µM.

Sedimentation-velocity analyses of the dimers of CAKβ/Pyk2 and its FERM domain in sucrose-density gradient containing either EGTA or Ca^{2+}/EGTA buffer with 500 nM free Ca^{2+}
A clarified cell lysate in 200 µl of lysis buffer without EDTA and sodium orthovanadate was prepared from HEK-293T cells expressing either FLAG-tagged wild-type CAKβ or FLAG-tagged FERM domain of the wild-type CAKβ. After addition of CaCl2 at 10 mM and incubation at 4°C for 1 h, the lysates of CAKβ and FERM domain were overloaded on 4.4 ml of a 15–50% stepwise sucrose-density gradient (8 steps of 0.55 ml each) and 1.98 ml of a 10–50% stepwise sucrose-density gradient (9 steps of 220 µl each) respectively, both prepared in 50 mM Hepes (pH 7.5), 150 mM NaCl, and either 2 mM EGTA or Ca^{2+}/EGTA (2 mM) buffer with 500 nM free-Ca^{2+} and subjected to sedimentation-velocity analyses at 1.8 × 10^5 g and 1.7 × 10^7 g respectively, for 17 h at 4°C [MCS50 rotor (Beckman) and SS55S rotor (Hitachi) respectively]. The Ca^{2+}/EGTA buffer was prepared according to the MaxChelator program. After centrifugation, 31% of 150 µl each or 31 fractions of 70 µl each were collected from the top to the bottom of each tube. Then 50 µl or 25 µl of a 4-fold SDS sample buffer was added to each fraction, which was then subjected to SDS/PAGE on 6% (CAKβ) or 10% (FERM domain) gels after heating at 70°C for 10 min. The separated proteins were analysed by Western blotting with anti-FLAG M2 antibody. BSA (67 kDa), rabbit muscle aldolase (tetramer, 158 kDa), bovine liver catalase (tetramer, 232 kDa) and horse spleen ferritin (24-mer, 440 kDa) were used as molecular-mass standards in the sedimentation-velocity analysis.

RESULTS
Calmodulin inhibitors blocked vasopressin-stimulated tyrosine phosphorylation of endogenous CAKβ/Pyk2 in WFB cells
We previously found that a rat fibroblast line, WFB, had endogenous CAKβ/Pyk2 [1] and that the stimulation of WFB cells with vasopressin or PDGF induced an increase in the cytoplasmic free-Ca^{2+} concentration and the hydrolysis of inositol lipids by phospholipase C [4]. The tyrosine phosphorylation of endogenous CAKβ/Pyk2 in WFB cells was enhanced by stimulating the cells with vasopressin, PDGF or the Ca^{2+} ionophore A23187 (Figure 1A). The vasopressin-stimulated tyrosine phosphorylation of CAKβ/Pyk2 was reduced by addition of BAPTA-AM, a cell-permeable Ca^{2+} chelator, to the WFB cells (Figure 1B), indicating that the enhanced tyrosine phosphorylation was dependent on the increase in the cytoplasmic free-Ca^{2+} concentration. As shown in Figure 1(C), calmodulin inhibitors [18,19] W7 and W13 blocked the vasopressin-stimulated tyrosine phosphorylation of CAKβ/Pyk2 in WFB cells, whereas their inactive analogues, W5 and W12 [18,19], did not.

CAKβ/Pyk2 formed a complex with Ca^{2+}/calmodulin, but FAK did not
The findings shown in Figure 1 prompted us to explore the possibility of CAKβ/Pyk2 binding to Ca^{2+}/calmodulin. As shown in Figure 2(A) (lanes 5–8), Myc-tagged CAKβ/Pyk2 in the cell lysate of transfected HEK-293T cells was trapped by calmodulin-agarose beads in the presence of 5 mM CaCl2 but not in the presence of 10 mM EGTA. Agarose beads used as a control did not bind CAKβ/Pyk2 in the presence or absence of CaCl2. Myc-tagged FAK expressed in transfected HEK-293T cells did not bind to Ca^{2+}/calmodulin-agarose (Figure 2A, lanes 9–12). This result was consistent with the known property of FAK not being regulated by Ca^{2+} signals. The binding of CAKβ/Pyk2 to Ca^{2+}/calmodulin was not affected by the tyrosine phosphorylation of CAKβ/Pyk2 because both Y402F (autophosphorylation-defective) and K457A (kinase-minus) mutants of CAKβ/Pyk2 bound to Ca^{2+}/calmodulin in a similar manner as wild-type CAKβ/Pyk2 (results not shown). Formation of a complex between endogenous CAKβ/Pyk2 and endogenous
Ca\(^{2+}\)/calmodulin was confirmed by the use of a cell lysate from HeLa cells (Figure 2B). Calmodulin was specifically co-immunoprecipitated with CAK\(β\)/PYK2 from the cell lysate in the presence of 1 mM CaCl\(_2\) (Figure 2B, lane 2). Calmodulin was not co-immunoprecipitated with FAK under the same conditions (Figure 2B, lane 3).

Ca\(^{2+}\)/calmodulin binds to the \(α\)-helix present in the FERM F2 subdomain of CAK\(β\)/PYK2

Next we tried to find the binding site of Ca\(^{2+}\)/calmodulin in CAK\(β\)/PYK2. A plasmid expressing the FERM domain (amino acid residues 37–356) of CAK\(β\)/PYK2 fused to the C-terminal end of the 3 × FLAG-tag was constructed (Figure 3A). This fusion protein present in the cell lysate from transfected HEK-293T cells specifically bound to Ca\(^{2+}\)/calmodulin-agarose in a similar manner to CAK\(β\)/PYK2 (Figure 3B, second panel). The result indicated that the CAK\(β\)/PYK2/CAK\(β\)/PYK2 complex contained the binding site for Ca\(^{2+}\)/calmodulin.

At an \(α\)-helix (F2-\(α\)-2) in the F2 subdomain (amino acid residues 135–260) of the CAK\(β\)/PYK2 FERM domain [6], we found a reverse basic 1–8–14 motif of the calmodulin target sequence. In Figure 4(A), this reverse basic 1–8–14 motif in amino acid residues 166–191 of CAK\(β\)/PYK2 is aligned with the known calmodulin target sequences of this motif. In the reverse
A FERM domain respectively. (proline-rich region; FERM-F1, FERM-F2, and FERM-F3, F1, F2, and F3 subdomains of the plasmids expressing Myc-tagged calmodulin (Myc-CaM), HA-tagged CAK β, and the FAK F2 subdomain (CFC-FERM), all of which were fused to the C-terminal end of 33–352; FAK-FERM) and a chimaeric FERM domain with the CAK β/FERM domain (amino acid residues 37–356; CAK β/PYK2 (β-amino acid residues 37–356; CAK β/ PYK2) Plasmids expressing the wild-type CAK β/ PYK2, the FAK FERM domain (amino acid residues 33–352; FAK-FERM), and a chimaeric FERM domain with the CAK β/FERM domain (Figure 3A, CFC-FERM) with the CAK β/ PYK2 F1 and F3 subdomains and the FAK F2 subdomain did not bind to Ca2+/calmodulin-agarose (Figure 3B, third panel). This result supports the assumption that the FERM F2 subdomain of CAK β/ PYK2 has the binding site for Ca2+/calmodulin. The reverse basic 1–8–14 motif found in CAK β/ PYK2 was a simple motif with conserved hydrophobic amino acid residues at positions 1, 7, and 14, and with two additional C-terminal arginine residues. The motif itself was also conserved in FAK at amino acid residues 163–178 (Figure 4B). However, consistent with the property of FAK not being regulated by Ca2+ signals, FAK did not form a complex with Ca2+/calmodulin (Figures 2 and 3B). The two serine residues in CAK β/ PYK2 at positions 13 and 16 in this motif (Ser168 and Ser171) are replaced in FAK by aspartic acid residues (Asp161 and Asp164) (Figure 4B). Presumably, these replacements by acidic residues may prevent binding of Ca2+/calmodulin to FAK.

If the binding of Ca2+/calmodulin to CAK β/ PYK2 was a simple motif with conserved hydrophobic amino acid residues at positions 1, 7, and 14, and with two additional C-terminal arginine residues. The motif itself was also conserved in FAK at amino acid residues 163–178 (Figure 4B). However, consistent with the property of FAK not being regulated by Ca2+ signals, FAK did not form a complex with Ca2+/calmodulin (Figures 2 and 3B). The two serine residues in CAK β/ PYK2 at positions 13 and 16 in this motif (Ser168 and Ser171) are replaced in FAK by aspartic acid residues (Asp161 and Asp164) (Figure 4B). Presumably, these replacements by acidic residues may prevent binding of Ca2+/calmodulin to FAK.

As will be shown later in Figures 6 and 7, the FERM domain of CAK β/ PYK2 formed a dimer after Ca2+/calmodulin binding. Myc-tagged calmodulin was co-precipitated with the dimer of the CAK β/ PYK2 FERM domain (Figure 3C) when Myc-tagged calmodulin and CAK β/ PYK2 FERM domains, tagged with either HA or FLAG, were triply co-expressed in HEK-293T cells and the FLAG-tagged FERM domain was immunoprecipitated from the cell lysate in the presence of CaCl2. The result is in accordance with a model in which Ca2+/calmodulin binding to the FERM domain induces dimer formation.

The FERM domain of CAK β/ PYK2 has the binding site for Ca2+/calmodulin

Figure 3 The FERM domain of CAK β/ PYK2 has the binding site for Ca2+/calmodulin

(A) Plasmids expressing the wild-type CAK β/ PYK2 (CAK β), the CAK β/ PYK2 FERM domain (amino acid residues 37–356; CAK β-FERM), the FAK FERM domain (amino acid residues 33–352; FAK-FERM) and a chimaeric FERM domain with the CAK β/ PYK2 F1 and F3 subdomains and the FAK F2 subdomain (CFC-FERM), all of which were fused to the C-terminal end of the 3 × FLAG-tag (FLAG), were constructed. Kinase, protein-tyrosine kinase domain; Pro, proline-rich region; FERM-F1, FERM-F2, and FERM-F3, F1, F2, and F3 subdomains of the FERM domain respectively. (B) Growing HEK-293T cells (1 × 10⁶ cells) were transfected with a plasmid expressing 3 × FLAG-tagged CAK β-FERM, CFC-FERM or FAK-FERM by the use of HilyMax™/Lipofectamine Plus™. The clarified cell lysate (100 µg of each protein) prepared in lysis buffer without EDTA was subjected to affinity precipitation for 2 h at 4°C with calmodulin-agarose (CaM beads) in the presence of either 5 mM CaCl2 (lane 2) or 10 mM EGTA (lane 3) as indicated. The bound proteins and the total cell lysate (5 µg of each protein; lane 1) were subjected to SDS/PAGE in a 9% gel, after which each FERM domain was visualized by blotting with an anti-FLAG antibody. The reproducibility of the result was confirmed by three separate experiments. (C) Co-immunoprecipitation of calmodulin with the dimer of CAK β-FERM domain. Growing HEK-293T cells were transfected using HilyMax™/Lipofectamine Plus™ with plasmids expressing Myc-tagged calmodulin (Myc-CaM), HA-tagged CAK β FERM domain (HA-FERM) and FLAG-tagged CAK β FERM domain (FLAG-FERM) as indicated. The clarified cell lysate (100 µg of each protein) prepared in lysis buffer without EDTA was subjected to immunoprecipitation (IP: FLAG) for 2 h at 4°C with anti-FLAG antibody in the presence of 5 mM CaCl2. The precipitated proteins and the total cell lysate (5 µg of each protein) were subjected to SDS/PAGE in a 9% gel, after which calmodulin and FERM domains were made visible by blotting with anti-Myc, anti-HA and anti-FLAG antibodies. The reproducibility of the result was confirmed by three separate experiments.

Binding of Ca2+/calmodulin to CAK β/ PYK2 was required for tyrosine phosphorylation of CAK β/ PYK2

If the binding of Ca2+/calmodulin to the α-helix (F2–o2) present in the F2 subdomain of the CAK β/ PYK2 FERM domain is important for the Ca2+-induced activation of CAK β/ PYK2, then the LQ/AA double-mutant CAK β/ PYK2 should be defective in the Ca2+-induced tyrosine phosphorylation of CAK β/ PYK2. As shown in
Figure 4 The LQ/AA double mutant of CAKβ/PYK2 bound to Ca\(^{2+}\)/calmodulin-agarose much less than the wild-type CAKβ/PYK2.

(A) CAKβ/PYK2 has a reverse basic 1-8-14 motif of the calmodulin target sequence at amino acid residues 170–183 in the FERM F2 subdomain. The reverse basic 1–8-14 motif of CAKβ/PYK2 found in amino acid residues 166–191 is aligned with the known calmodulin target sequences of the reverse basic 1–8–14 motif. c, coiled region; H, α-helix; hCAKβ, human CAKβ [2]; rCAKβ, rat CAKβ [1]; zpFTK2B, zebrafish CAKβ (NCBI NP 997735); HIV1–gp160, gp160 of HIV-1 [21]; rCaMKKα, rat CaMKKα [20]; cCaMKKα, Caenorhabditis elegans Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase [20]. (B) The reverse basic 1–8–14 motif of human FAK (hFAK) found in amino acid residues 159–164 is aligned with that of human CAKβ/PYK2 (nCAKβ). Note that the Ser\(^{148}\) and Ser\(^{151}\) residues of CAKβ/PYK2 are replaced by corresponding acidic residues, Asp\(^{118}\) and Asp\(^{148}\), in FAK. (C) Growing HEK-293T cells (1 × 10\(^5\) cells) were transfected with a plasmid expressing either 3 × FLAG-tagged CAKβ (WT-CAKβ; lanes 3–7), 3 × FLAG-tagged LQ/AA-mutant CAKβ (LQ/AA-CAKβ; lanes 8–12) or an empty vector (vector; lanes 1 and 2) by the use of Lipofectamine Plus™. At 28 h after transfection, the cells were cultured in the absence of serum for an additional 14 h. The end of the serum-free culture, the cells were exposed, where indicated, to 100 μM BAPTA-AM for 30 min (lanes 5 and 10), 50 μM W7 (a calmodulin inhibitor) for 2 h (lanes 6 and 11) or 50 μM W5 (an inactive analogue of W7) for 2 h (lanes 7 and 12). Where indicated, the cells were stimulated with 1 μM A23187 for 10 min (lanes 2, 4 and 9). The clarified cell lysates prepared in lysis buffer were subjected to immunoprecipitation with an anti-FLAG antibody at 4 °C for 2 h (IP: FLAG). The precipitated proteins were separated by SDS/PAGE in a 6 % gel. The tyrosine phosphorylation of CAKβ/PYK2 was made visible by blotting with antibody 4G10 (blot: pTyr), after which an equal loading of CAKβ/PYK2 in each lane was verified by reprobing with an anti-FLAG antibody (blot: FLAG) after stripping off the 4G10 antibody. The values (means ± S.D. (n = 5)) given at the bottom of each lane represent relative densities of each band blotted with anti-pTyr, in which the ratios of band densities between the anti-pTyr blot and anti-FLAG blot were normalized by setting the ratio of lane 3 at 1.00. The reproducibility of the result was confirmed by five separate experiments.

Figure 5 This was exactly the case. In this experiment, either FLAG-tagged wild-type CAKβ/PYK2 or FLAG-tagged LQ/AA-mutant CAKβ/PYK2 was expressed in WFB cells. The wild-type CAKβ/PYK2 expressed in WFB cells was significantly tyrosine phosphorylated without stimulation from outside, and this basal tyrosine phosphorylation at high levels was strongly suppressed by adding either BAPTA-AM (a cell-permeable Ca\(^{2+}\)-chelator) or W7 (a calmodulin inhibitor) to the culture medium (Figure 5, lanes 3 and 5–7). In the LQ/AA-mutant CAKβ/PYK2 expressed in WFB cells, the level of tyrosine phosphorylation was quite low (Figure 5, lane 8). These results indicated that the tyrosine phosphorylation of the wild-type CAKβ/PYK2 in the overexpressed cells without stimulation from outside was also dependent on Ca\(^{2+}\)/calmodulin and that the Ca\(^{2+}\)/calmodulin present in cells not evidently stimulated with Ca\(^{2+}\)-mobilizing extracellular ligands participated in maintaining the tyrosine phosphorylation of the overexpressed wild-type CAKβ/PYK2 at a high level. The overexpressed LQ/AA-mutant CAKβ/PYK2 was markedly defective in this activation by Ca\(^{2+}\)/calmodulin present in unstimulated cells (Figure 5, lane 8 and 10–12). The tyrosine phosphorylation of the overexpressed wild-type CAKβ/PYK2 was enhanced only to a limited extent on stimulation with the Ca\(^{2+}\)-ionophore A23187 (Figure 5, lane 9), indicating that the overexpressed wild-type CAKβ/PYK2 was almost fully activated without stimulating cells from outside. The overexpressed wild-type CAKβ/PYK2 was fully tyrosine-phosphorylated at residue 402 without stimulation from outside; no significant increase in staining of the protein with anti-phospho-Pyk2 (Tyr\(^{402}\)) antibody was found after the stimulation with A23187 (results not shown). The low tyrosine phosphorylation of the LQ/AA-mutant CAKβ/PYK2 in WFB cells was not significantly enhanced on stimulation with the Ca\(^{2+}\)-ionophore (Figure 5, lane 9). The same results were obtained when HeLa cells were used in the experiment shown in Figure 5 in place of WFB cells (results not shown).

Figure 5 The LQ/AA double-mutant CAKβ/PYK2 overexpressed in WFB cells was markedly less tyrosine phosphorylated than the overexpressed, wild-type CAKβ/PYK2, the tyrosine phosphorylation of which was dependent on Ca\(^{2+}\)/calmodulin present in cells without evident stimulation from outside.

Growing WFB cells (1 × 10\(^5\) cells) were transfected with plasmid expressing either 3 × FLAG-tagged CAKβ (WT-CAKβ; lanes 3–7), 3 × FLAG-tagged LQ/AA-mutant CAKβ (LQ/AA-CAKβ; lanes 8–12) or an empty vector (vector; lanes 1 and 2) by the use of Lipofectamine Plus™. At 28 h after transfection, the cells were cultured in the absence of serum for an additional 14 h. The end of the serum-free culture, the cells were exposed, where indicated, to 100 μM BAPTA-AM for 30 min (lanes 5 and 10), 50 μM W7 (a calmodulin inhibitor) for 2 h (lanes 6 and 11) or 50 μM W5 (an inactive analogue of W7) for 2 h (lanes 7 and 12). Where indicated, the cells were stimulated with 1 μM A23187 for 10 min (lanes 2, 4 and 9). The clarified cell lysates prepared in lysis buffer were subjected to immunoprecipitation with an anti-FLAG antibody at 4 °C for 2 h (IP: FLAG). The precipitated proteins were separated by SDS/PAGE in a 6 % gel. The tyrosine phosphorylation of CAKβ/PYK2 was made visible by blotting with antibody 4G10 (blot: pTyr), after which an equal loading of CAKβ/PYK2 in each lane was verified by reprobing with an anti-FLAG antibody (blot: FLAG) after stripping off the 4G10 antibody. The values (means ± S.D. (n= 5)) given at the bottom of each lane represent relative densities of each band blotted with anti-pTyr, in which the ratios of band densities between the anti-pTyr blot and anti-FLAG blot were normalized by setting the ratio of lane 3 at 1.00. The reproducibility of the result was confirmed by five separate experiments.

The FERM domain of CAKβ/PYK2 formed a dimer that was Ca\(^{2+}\)-dependent and was markedly reduced in the LQ/AA double mutant.

The receptor protein-tyrosine kinases are activated by dimerization and transphosphorylation following ligand binding [22]. FAK and CAKβ/PYK2 might also be activated by dimer
in each incubation mixture was determined by the fluorescence of Fluo-4 added at 4 µM in each lysate prepared for this determination with and without added CaCl2. The results indicated that the free-Ca\(^{2+}\) concentration in each incubation mixture increased from 1.8 µM to 12.8 µM, corresponding to the increase in the added CaCl2 from 0 to 20 mM (Figure 6B). Thus the results on the LQ/AA mutant and the dependence on the free-Ca\(^{2+}\) concentration shown in Figure 6 suggested that the dimer formation of the CAKβ/PYK2 FERM domain was dependent on Ca\(^{2+}\)/calmodulin-binding to the region containing Leu\(^{176}\) and Gln\(^{177}\).

**CAKβ/PYK2 FERM domain formed a dimer by binding of Ca\(^{2+}\)/calmodulin to an α-helix present at the FERM F2 subdomain**

The Ca\(^{2+}\)-dependent dimer formation of the CAKβ/PYK2 FERM domain was inhibited by a calmodulin inhibitor (W7) but not by its inactive-analogue (W5) (Figure 7A). Addition of W7 at final concentrations of 50 µM and 100 µM to the incubation mixture containing both HA-tagged and FLAG-tagged CAKβ/PYK2 FERM domains followed by immunoprecipitation with an anti-HA antibody resulted in the reduction of the co-precipitated FLAG-tagged FERM domain to 49% and 35% respectively (Figure 7B). The dimer formation was also inhibited by the synthetic peptide with the amino acid sequence at residues 168–188 of CAKβ/PYK2 but not by the peptide with its LQ/AA double-mutant sequence (Figure 7C). Addition of the peptide with the wild-type sequence at final concentrations of 30 µM and 60 µM to the incubation mixture containing both HA-tagged and FLAG-tagged CAKβ/PYK2 FERM domains resulted in reduction of the co-precipitated FLAG-tagged FERM domain to 81% and 47% respectively (Figure 7D). These results strongly indicate that the dimer formation of the CAKβ/PYK2 FERM domain was Ca\(^{2+}\)/calmodulin-dependent and that the amino acid residues in CAKβ/PYK2 FERM domain containing Leu\(^{176}\) and Gln\(^{177}\) were the binding site for Ca\(^{2+}\)/calmodulin. Thus the binding of Ca\(^{2+}\)/calmodulin to the α2-helix (F2-α2) (amino acid residues 172–186) [6] present in the FERM F2 subdomain of CAKβ/PYK2 induced dimer formation of the FERM domain.

Lipskis et al. [15] has already shown that co-expression of the CAKβ/PYK2 FERM domain with wild-type CAKβ/PYK2 strongly reduced the tyrosine phosphorylation of the overexpressed CAKβ/PYK2 in SF767 cells, a human glioblastoma line. We confirmed in HeLa cells the result of Lipskis et al. [15]; the expression of the HA-tagged CAKβ/PYK2 FERM domain in HeLa cells markedly reduced the tyrosine phosphorylation of CAKβ/PYK2, an inhibition highly likely resulting from the formation of the heterodimer between the FERM domain and CAKβ/PYK2. We suppose that the heterodimer formation in the overexpressing cells is triggered by Ca\(^{2+}\)/calmodulin present in cells not evidently stimulated with Ca\(^{2+}\)-mobilizing ligands.

**Sedimentation-velocity analyses of CAKβ/PYK2 and its FERM domain; their dimers were stable in 500 nM free Ca\(^{2+}\) and dissociated in 2 mM EGTA**

We found that CAKβ/PYK2 formed a stable dimer when CaCl2 was added at 10 mM or more to the lysate from HEK-293T cells in which FLAG-tagged CAKβ/PYK2 and Myc-tagged CAKβ/PYK2 were co-expressed (results not shown). The formation of the stable dimer in the presence of CaCl2 was quite different from the transient, weak formation of the CAKβ/PYK2 dimer shown by Park et al. [23], in which the Ca\(^{2+}\) concentration...
The FERM domain was then immunoprecipitated with an anti-HA antibody, \( \beta \)168–188 of CAK sequence. Growing HEK-293T cells (1 \( \mu \) concentration (0–60 concentration (10–100 concentration (10–100 µM) were doubly transfected with plasmids each expressing the HA-tagged or FLAG-tagged FERM domain (FLAG-FERM, HA-FERM) of the wild-type CAKβ/PYK2 by the use of HilyMaxTM. Control cells (lanes 1 and 2) were transfected with an empty vector (vector). At 48 h after the transfection, clarified cell lysates were prepared as in Figure 6(B). The FLAG-tagged dimer of the CAKβ/PYK2 FERM domain formed in the lystate in the presence of 10 mM CaCl\(_2\) was overlaid on sucrose-density gradients prepared in buffers containing either 2 mM EGTA or Ca\(^{2+}\)/EGTA (2 mM) buffer of 500 nM free Ca\(^{2+}\) and subjected to a sedimentation-velocity analysis (Figure 8A). The FLAG-tagged CAKβ/PYK2 migrated mostly as a monomer, the peak at around fraction 9, in the sucrose-density gradient containing 2 mM EGTA (Ca\(^{2+}\)-free buffer) (Figure 8A). In the sedimentation of the FLAG-tagged CAKβ/PYK2 in sucrose-density gradient containing Ca\(^{2+}\)/EGTA buffer of 500 nM free Ca\(^{2+}\), an obvious shift towards a higher molecular mass was observed, in which almost no monomer was found and an increase in the FLAG-tagged CAKβ/PYK2 at approx. fraction 16 was found (Figure 8A). The dimer complexed with calmodulin at a 1:1 molar ratio was expected to migrate at approx. fraction 16. In these sedimentation-velocity analyses, a complex of the FLAG-tagged CAKβ/PYK2 with a protein of 40–60 kDa was observed at approx. fraction 12. No positive evidence for the formation of trimer or higher oligomers was found.

Although a large amount of the complex formed between the FLAG-tagged CAKβ/PYK2 and a protein of 40–60 kDa obscured the formation of the dimer at approx. fraction 16 in the experiment shown in Figure 8(A), we were able to show unambiguously a Ca\(^{2+}\)-dependent dimer formation of the FLAG-tagged FERM domain of CAKβ/PYK2 (Figure 8B). The FLAG-tagged dimer of the CAKβ/PYK2 FERM domain formed in the lystate in the presence of 10 mM CaCl\(_2\) was overlaid on sucrose-density gradients containing either 2 mM EGTA or Ca\(^{2+}\)/EGTA (2 mM) buffer of 500 nM free Ca\(^{2+}\) and subjected to sedimentation-velocity analysis (Figure 8B). In the sucrose-density gradient containing 2 mM EGTA, the FLAG-tagged FERM domain migrated mostly as a monomer at approx. fraction 9–10 (Figure 8B). In the sucrose-density gradient containing Ca\(^{2+}\)/EGTA buffer of 500 nM free Ca\(^{2+}\), the migration of all the FLAG-tagged FERM domain shifted towards a higher molecular mass with a peak at fractions 14–15 (Figure 8B), where the dimer complexed with calmodulin at a 1:1 molar ratio was expected to migrate. No significant peak was found at fraction 18, where the trimer was expected. These results support the conclusion that free Ca\(^{2+}\) at 500 nM was enough to prevent the CAKβ/PYK2 dimer from dissociating into monomer. The CAKβ/PYK2 dimer dissociated into monomer in the Ca\(^{2+}\)-free buffer containing 2 mM EGTA. Thus the Ca\(^{2+}\)-concentration dependency of the CAKβ/PYK2 dimerization is in accordance with the assumption that CAKβ/PYK2 dimerizes following the binding of Ca\(^{2+}\)/calmodulin to the protein.

**DISCUSSION**

It is known that CAKβ/PYK2 is a unique protein-tyrosine kinase activated following an increase in the cytoplasmic free-Ca\(^{2+}\) concentration. However, the mechanism underlying this
Figure 8  Sedimentation-velocity analyses showed that the dimers of CAKβ/PYK2 and its FERM domain were stable in 500 nM free Ca2+ and dissociated in 2 mM EGTA to their monomers.

Growing HEK-293T cells (5 x 10^5 cells) were transfected with a plasmid expressing either FLAG-tagged wild-type CAKβ (A) or FLAG-tagged its FERM domain (B) by the use of HilyMaxTM. At 48 h after transfection, the cell lysates were prepared in 200 µl of lysis buffer without EDTA and sodium orthovanadate. The clarified cell lysates were prepared, incubated for 1 h at 4°C after addition of CaCl2 at 10 mM, and then overlaid on sucrose-density gradients (4.4 ml in A and 1.98 ml in B) containing either 2 mM EGTA or Ca2+/EGTA (2 mM) buffer of 500 nM free-Ca2+ concentration for the sedimentation-velocity analyses as described in the Experimental section. After centrifugation, 31 fractions of 150 µl each (A) or 70 µl each (B) were collected from the top to the bottom of a tube. Each fraction was subjected to SDS/PAGE analyses for FLAG-tagged CAKβ (A) and its FERM domain (B). The figures given in the vertical axis represent the amount (percentage of total) of FLAG-tagged CAKβ (A) or its FERM domain (B) contained in each fraction. From the sedimentation-velocities of marker proteins shown in the lower panel of (A), the FLAG-tagged CAKβ monomer, and its dimer and trimer complexed with calmodulin at a 1:1 molar ratio were estimated to move down to fractions 9, 16 and 21–22 respectively. The sedimentation-velocities of marker proteins shown in the lower panel of (B) indicate that the FLAG-tagged monomer of the CAKβ FERM domain, and its dimer and trimer complexed with calmodulin at a 1:1 molar ratio moved down to fractions 9–10, 15 and 18 respectively. Experiments (A and B) were repeated twice each to confirm the reproducibility. MW, molecular mass x 10^-3; CBB, Coomassie Brilliant Blue.

Ca2+ sensitivity of CAKβ/PYK2 has remained unknown. The activation of CAKβ/PYK2 by binding of Ca2+/calmodulin reported in the present study is the simplest mechanism for the regulation of CAKβ/PYK2 by Ca2+. Calmodulin is the predominant intracellular receptor for Ca2+ with the function of a Ca2+ sensor. To the best of our knowledge, all Ca2+/calmodulin-dependent protein kinases known so far are serine/threonine protein kinases [24]. CAKβ/PYK2 is the first protein-tyrosine...
kinase regulated by Ca\(^{2+}\)/calmodulin. We suppose that the absence of a typical Ca\(^{2+}\)/calmodulin-binding motif in CAK/β/PYK2 may possibly be the reason why the activation of CAK/β/PYK2 by Ca\(^{2+}\)/calmodulin was not found until now. The Ca\(^{2+}\)/calmodulin-binding sequence in CAK/β/PYK2 identified in the present study is rare in being a reverse basic 1-8-14 motif, in which the binding orientation is opposite to that observed in most other known calmodulin-target sequences. Ca\(^{2+}\)/calmodulin binding to a reverse basic 1-8-14 motif was first described by Osawa et al. [20] in CaMKK. We found that FAK did not bind Ca\(^{2+}\)/calmodulin, a result consistent with the known property of FAK not being regulated by Ca\(^{2+}\) signals. The two serine residues in the reverse basic 1-8-14 motif of CAK/β/PYK2 were replaced in FAK by aspartic acid residues; these replacements by acidic residues probably prevent binding of Ca\(^{2+}\)/calmodulin to FAK.

Cecarelli et al. [6] reported the crystal structure of the FERM domain of FAK. The amino acid sequences of the FERM domains in human FAK and CAK/β/PYK2 are 45.6% identical and these two FERM domains have, without doubt, the same overall structure. The Ca\(^{2+}\)/calmodulin-binding sequence in CAK/β/PYK2, the reverse basic 1-8-14 motif, is located at an α-helix (F2-α2) present in the outer portion of the FERM domain [6]. Next to this α-helix, there is another α-helix (F2-α3) containing a large patch of basic residues, K\(^{223}\)PKQFRK\(^{229}\). This α-helix (F2-α3) with basic residues in the FERM F2 subdomain is also present in FAK, in which it was shown to be important in cell-adhesion-dependent activation of FAK and downstream signalling [11]. The hepatocyte growth factor receptor c-Met directly binds to these basic residues of the FAK FERM F2 subdomain at its phosphorylated cytoplasmic tyrosine residue(s), resulting in the activation of FAK. This activation of FAK by c-Met leads to hepatocyte growth factor-induced cell motility and cell invasion [14]. These two α-helices, F2-α2 and F2-α3, in the FERM F2 subdomain are present at the external portion of the FAK and CAK/β/PYK2 FERM domain [6], making easy access possible to these α-helices from outside by regulatory proteins such as Ca\(^{2+}\)/calmodulin. Recently, the structural basis for the autoinhibition and activation of FAK was revealed by the crystal structure [13]. In the autoinhibited state, FAK is locked in an inactive conformation, in which a direct contact of the C-lobe of the kinase domain to a hydrophobic pocket on the FERM F2 lobe formed by Phe\(^{396}\)/Met\(^{398}\), Val\(^{399}\) and Leu\(^{401}\) is most important. The two α-helices, F2-α2 and F2-α3, are immediately adjacent to the hydrophobic pocket in the FERM F2 lobe [6]. As we mentioned above, the F2-α3 helix with the conserved basic patch has been postulated to represent an initial site of docking for an activating protein such as c-Met. The binding of an activating protein to the F2-α3 helix might then disrupt the FERM/kinase interface to activate FAK [13]. The F2-α2 helix and the F2-α3 helix are present next to the hydrophobic pocket, which binds to Phe\(^{396}\) on the FAK kinase C-lobe. The hydrophobic pocket on the FERM F2 lobe and the phenylalanine residue on the kinase C-lobe are also conserved in CAK/β/PYK2. Therefore it is tempting to speculate that the Ca\(^{2+}\)/calmodulin binding to the FERM F2-α2 helix of CAK/β/PYK2 might somehow disrupt the binding of FERM of the kinase domain to activate CAK/β/PYK2. It is possible that the Ca\(^{2+}\)/calmodulin binding to the FERM F2-α2 helix of CAK/β/PYK2 is directly linked to the formation of CAK/β/PYK2 dimer. However, it is also possible that the binding first liberates the kinase domain from inhibition by the FERM domain before dimerization.

We repeatedly found in WFB, HeLa and other cells that CAK/β/PYK2 was strongly tyrosine phosphorylated at its Tyr\(^{402}\) when it was exogenously overexpressed in cells from transfected plasmids. In this phenotype, exogenously overexpressed CAK/β/PYK2 is different from the endogenous protein, the tyrosine phosphorylation of which can be observed only after cells are stimulated with signals from outside. Results published by others also confirm this point [12,15,23]. FAK is different from CAK/β/PYK2 in this property: exogenously overexpressed FAK is only moderately tyrosine phosphorylated in a similar manner to endogenous FAK [12,15]. The reason for this enhanced tyrosine phosphorylation of exogenously overexpressed CAK/β/PYK2 remains to be explained. In our experiments, one of which is shown in Figure 5, we found the underlying mechanism for this enhanced tyrosine phosphorylation. The enhanced tyrosine phosphorylation of exogenously overexpressed CAK/β/PYK2 in WFB cells was strongly suppressed by treating cells with BAPTA-AM, a cell-permeable Ca\(^{2+}\) chelator, or W7, a calmodulin inhibitor, but not with its inactive analogue, W5 (Figure 5). Moreover, a mutant CAK/β/PYK2 with a defect in the Ca\(^{2+}\)/calmodulin binding at the reverse basic 1-8-14 motif in the FERM F2 subdomain, the LO/AA double mutant, was almost defective in this enhanced tyrosine phosphorylation when exogenously overexpressed in cells (Figure 5). From these results, we concluded that CAK/β/PYK2 exogenously overexpressed in cells from a transfected plasmid was tyrosine phosphorylated by binding of Ca\(^{2+}\)/calmodulin present in cells without evident stimulation with Ca\(^{2+}\)-mobilizing extracellular ligands. In living cells, the cytosolic free-Ca\(^{2+}\) concentration fluctuates locally within the cell, and transiently from time to time, as shown, for example, in the study on Ca\(^{2+}\) lightning caused by cell–cell contacts [5]. Thus some Ca\(^{2+}\)/calmodulin is always formed within a cell, which may trigger the dimer formation of overexpressed CAK/β/PYK2. These results also support the notion that Ca\(^{2+}\)/calmodulin-binding to the α2-helix of the FERM F2 subdomain is an essential step for activation of the wild-type CAK/β/PYK2. Activation of overexpressed tyrosine-kinases by forming spontaneous dimers in cells without stimulation from outside has already been shown in receptor tyrosine-kinases overexpressed in cancer cells [25], although Ca\(^{2+}\)/calmodulin is not involved in this case.

We have shown that the FERM domain of CAK/β/PYK2 formed a dimer in the presence of 8–12 μM free Ca\(^{2+}\) (Figures 6 and 7). The dimer was found to be stable in a buffer containing 500 nM free Ca\(^{2+}\), but dissociated into a monomer in a Ca\(^{2+}\)-free buffer containing 2 mM EGTA (Figure 8). The dimer formation of the FERM domain was dependent on the Ca\(^{2+}\)/calmodulin-binding to the FERM F2 subdomain, where amino acid residues Leu\(^{196}\) and Gin\(^{197}\) are found, is the binding site for Ca\(^{2+}\)/calmodulin. The expression of the CAK/β/PYK2 FERM domain in HeLa cells markedly reduced the tyrosine phosphorylation of co-expressed, wild-type CAK/β/PYK2. The result can be explained by the following model: the activation and transphosphorylation of CAK/β/PYK2 were blocked by heterodimer formation between the wild-type CAK/β/PYK2 and FERM domain in cells overexpressing them after binding of Ca\(^{2+}\)/calmodulin present in cells without evident stimulation from outside.

In the presence of 500 nM free Ca\(^{2+}\), CAK/β/PYK2 migrated as a dimer with a migration constant of 1.4 × 10\(^{-5}\) in a sedimentation-velocity analysis in a sucrose-density gradient, whereas in a Ca\(^{2+}\)-free buffer containing 2 mM EGTA, CAK/β/PYK2 migrated as a monomer (Figure 8). The results on the Ca\(^{2+}\)-concentration dependency of CAK/β/PYK2 dimerization are in accordance with our assumption that CAK/β/PYK2 requires Ca\(^{2+}\) as a ligand to calmodulin for the dimerization. Formation of the CAK/β/PYK2 dimer may induce a conformational change of the protein-tyrosine phosphorylation.
kinase, resulting in transphosphorylation within the dimer at Tyr402.

It is possible that the FERM domain of CAKβ/PYK2 has an intrinsic latent property to form a dimer by intermolecular interaction; the binding of Ca\(^{2+}/\text{calmodulin}\) is probably important as a trigger to form the dimer. However, it still remains unknown how CAKβ/PYK2 is autoinhibited, how the FERM domain is involved in this autoinhibition, and how the Ca\(^{2+}/\text{calmodulin}\) binding participates in liberating the kinase domain from the autoinhibition. The study on the mode of CAKβ/PYK2 activation may eventually lead to finding specific means to inhibit the CAKβ/PYK2 activity in cancer cells, in which CAKβ/PYK2 is often overexpressed and possibly involved in their spreading and invasion into surrounding tissues [15,25,26].

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