My Research on the Ca^{2+}/calmodulin-activated Protein-tyrosine Kinase, CAKβ/PYK2

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Here, I would like to describe a detailed summary of my lecture on Ca^{2+}/calmodulin-activated protein-tyrosine kinase, CAKβ/PYK2, given at Sapporo Medical University on Feb. 27, 2008, one month before my retirement.

Structure of FAK and CAKβ/PYK2, the two protein-tyrosine kinases of the FAK subfamily

We identified the second protein-tyrosine kinase of the FAK (focal adhesion kinase) subfamily and named the kinase CAKβ (Cell Adhesion Kinase β) (J. Biol. Chem. (1995) 270, 21206-21219). Almost at the same time, this protein kinase was published in Nature by the group of Schlessinger with a name of PYK2 (proline-rich tyrosine kinase 2). However, our paper was accepted for publication 3 months earlier than that of Schlessinger. The human genome has about 100 protein-tyrosine kinase genes, where FAK and CAKβ/PYK2 constitute one unique subfamily consisting of only these 2 members.

CAKβ/PYK2 contains 1009 amino acid residues, while FAK contains 1052. At the center of these proteins, there is a protein-tyrosine kinase domain of 261 amino acid residues, where 60% residues are identical between them. The skeletal 3-dimensional structure of all protein-tyrosine kinase domains is conserved and consists of N-lobe, activation loop, and C-lobe. Very close to the carboxyl end, each of FAK and CAKβ/PYK2 has a Focal Adhesion Targeting (FAT) region of 124 amino acid residues, where 62% residues are identical. We found that Hic-5, a protein of the paxillin superfamily, binds to the FAT of CAKβ/PYK2 and help to facilitate the localization of the protein at the focal adhesion (J. Biol. Chem. (1998) 273, 1003-1014). Near at the amino terminus, each of FAK and CAKβ/PYK2 has a FERM (band four-point-one, ezrin, radixin, moesin homology) domain of 320 amino acid residues, where 46% residues are identical and 18% residues are homologous between them. The FERM domain is a three-lobed domain consisting of F1-, F2-, and F3-subdomains as revealed by crystal structure. A Linker segment of 63 amino acid residues is present between the FERM and kinase domains of these proteins. In these Linker segments, there is a tyrosine residue autophosphorylated upon activation of these proteins, leading to the binding of the Src tyrosine kinase with its SH2 and SH3 domains and consequent full catalytic activation of Src. Between the kinase and FAT domains, each of these proteins has a region containing 2 polyproline type 2 helices, where signal transducing proteins bind with their SH3 domains generating downstream signals. The amino acid sequences of these proline-rich regions are only 23% identical between CAKβ/PYK2 and FAK.

Structural basis for autoinhibition and activation

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of FAK

FAK was discovered in 1992 as a protein tyrosine-phosphorylated by v-Src. The protein-tyrosine kinase activity of FAK is activated following the ligand-induced activation of integrin and some growth factor receptors regulating cellular responses including cell adhesion, migration, and survival (suppression of apoptosis). As was first shown in c-Src, each of, probably, all the normal protein-tyrosine kinases has an intramolecular mechanism autoinhibiting its protein-tyrosine kinase activity under the conditions without stimulation by an upstream signal. In FAK, it has been shown that the delation of its FERM domain activated the kinase activity. The research group of Schaller and Eck showed in 2007 by crystallography structural basis for the autoinhibition and activation of FAK (Cell (2007) 129, 1177-1187). In the autoinhibited state, FAK is locked in an inactive conformation, in which a direct binding of Phe^{596} on the FAK kinase C-lobe to a hydrophobic pocket on the FERM F2 lobe formed by Y^{180}, M^{183}, V^{196} and L^{197} is most important. This hydrophobic binding, which blocks access to the kinase catalytic cleft at the side of the kinase domain C-lobe, is strengthened by surrounding 4 hydrogen bonds formed between the C-lobe and the FERM F2-subdomain. Moreover, the tyrosine 397 at the autophosphorylation site in the Linker segment binds to the FERM F1-subdomain, thus closing the kinase active center also at the side of the kinase domain N-lobe. The FERM F2-subdomain α3 helix rich in basic amino acids (K^{216}, K^{218}, R^{221}, and R^{222}) has been postulated to represent an initial site of docking for an activating protein such as c-Met (the hepatocyte growth factor receptor). Binding of an activating protein to the F2-α3 helix might disrupt the hydrophobic binding pocket for the C-lobe F^{596} and unlocks the C-lobe to activate FAK. The release of the kinase C-lobe from the FERM F2-subdomain induces an autophosphorylation of Y^{397} at the Linker segment making the kinase N-lobe free from the FERM F1-subdomain and allowing the binding of Src at Y^{397}. The bound and activated Src then phosphorylates Y^{576} and Y^{577} at the activation loop of the kinase domain, thus fixing FAK in the active conformation.

Activation of the CAKβ/PYK2 protein-tyrosine kinase; binding of Ca^{2+}/calmodulin to its FERM F2 α2 helix followed by dimerization of the kinase

Tyrosine phosphorylation of CAKβ/PYK2 is enhanced after stimulation of cells with ligands such as vasopressin, PDGF, lysophosphatidic acid and endothelin that binds to receptors linked to phospholipase C activation. It has been known that the tyrosine-phosphorylation of CAKβ/PYK2, but not of FAK, is enhanced following an increase in cytoplasmic free Ca^{2+}-concentration. The mechanism underlying this Ca^{2+}-induced activation of CAKβ/PYK2 remained unknown since 1995. In 2007, we succeeded in showing how Ca^{2+} activates CAKβ/PYK2 (Biochem. J. (2008) 410: 513-523). Our article was published in the Biochemical Journal (Printed in Great Britain) together with an editorial commentary by Michael D. Schaller (Biochemical. J. (2008) 410: e3-e4), one of the 2 principal authors of the article on FAK summarized above. We found that the FERM F2-subdomain of CAKβ/PYK2 binds Ca^{2+}/calmodulin, which, presumably, resulted in the release of the CAKβ/PYK2 kinase domain C-lobe from binding by its F2-subdomain. The binding of Ca^{2+}/calmodulin to CAKβ/PYK2 induced a homodimer formation at the FERM domain, which might then induce a conformational change of the kinase domain, resulting in the transphosphorylation within the dimer of the tyrosine 402 at the Linker segment of CAKβ/PYK2. To the autophosphorylated Y^{402} and nearby polyproline type2 helix (PQIPT) at the Linker segment, c-Src binds with its SH2 and SH3 domains and then phosphorylates the 2 tyrosines (Y^{579} and Y^{580}) at the activation loop of the CAKβ/PYK2 kinase domain leading to the full activation of the protein-tyrosine kinase. Our model illustrated in Figure 1 is based on our following findings. Calmodulin inhibitors blocked the tyrosine

phosphorylation of endogenous CAKβ/PYK2 in cultured fibroblasts stimulated with vasopressin, PDGF, or A23187, a Ca^{2+}-ionophore. Calmodulin coimmunoprecipitated with CAKβ/PYK2, but not with FAK, from HeLa cell lysate. Calmodulin-agarose bound CAKβ/PYK2, but not FAK, in the presence of $CaCl_2$. A calmodulin-binding sequence of reverse basic 1-8-14 motif is found in the FERM F2-subdomain α2 helix (F2-α2) of CAKβ/PYK2. This α-helix (F2-α2) was found to be the binding site of Ca^{2+}/calmodulin; a mutant at this motif, L176A/Q177A (LQ/AA) CAKβ/PYK2, bound to Ca^{2+}/calmodulin much less than the wild-type. CAKβ/PYK2 is almost fully tyrosine phosphorylated when overexpressed in cells from cDNA; no significant increase in the tyrosine-phosphorylation was found after the stimulation with A23187. This basal tyrosine phosphorylation at high level was strongly suppressed by W7, an inhibitor of calmodulin, or by a cell-permeable Ca^{2+}-chelator. Tyrosine phosphorylation of the LQ/AA-mutant CAKβ/PYK2 overexpressed in fibroblasts was quite low and was not significantly enhanced on stimulation with Ca^{2+}-ionophore. Therefore, the tyrosine phosphorylation of the wild-type CAKβ/PYK2 in the overexpressed cell without stimulation from outside was also dependent on Ca^{2+}/calmodulin present at low level. The CAKβ/PYK2 FERM domain and also CAKβ/PYK2 itself formed homodimers on binding of Ca^{2+}/calmodulin. 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 or by a synthetic peptide with amino acid residues 168-188 of CAKβ/PYK2, but not by a peptide with its LQ/AA-mutant sequence. The hydrophobic pocket on the FAK FERM F2 lobe and the phenylalanine residue on the FAK kinase C-lobe trapped by the hydrophobic pocket are also conserved in CAKβ/PYK2. Therefore, it is tempting to speculate that the Ca^{2+}/calmodulin

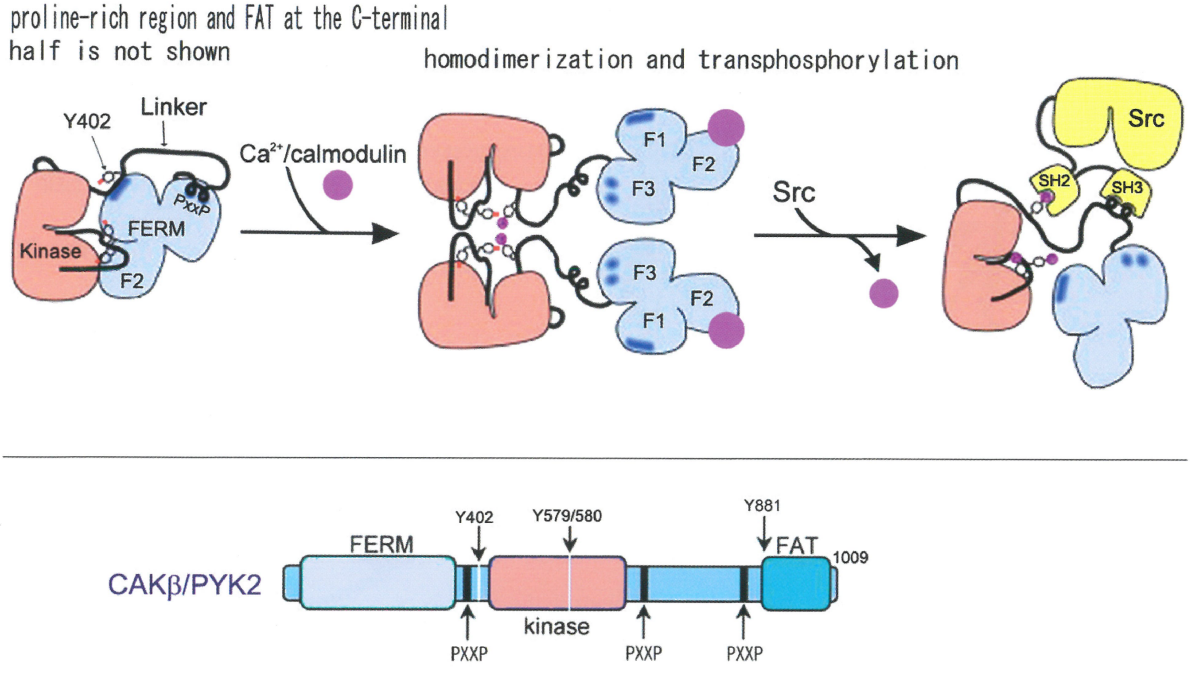

Figure 1 Protein-tyrosine kinase CAKβ/PYK2 is activated by binding Ca^{2+}/calmodulin to FERM F2-α2 helix and thus forming its dimer.

binding to the FERM F2-α2 helix of CAKβ/PYK2 might somehow disrupt the inhibitory binding of FERM to the kinase domain. The F2-α2 helix is present immediately adjacent to the hydrophobic pocket in the CAKβ/PYK2 FERM F2 lobe formed by F^{187}, M^{190}, L^{203} and L^{204}, which presumably locks 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 autoinhibiting FERM domain and activates the kinase by forming a dimer and by transphosphorylation within the dimer of Tyr^{402} at the Linker segment. It is possible that the Ca^{2+}/calmodulin binding to the FERM F2-α2 helix of CAKβ/PYK2 is directly linked to the formation of CAKβ/PYK2 dimer. In FAK, the cytoplasmic region of ligand-activated integrin or growth factor receptors frees the kinase C-lobe in the autoinhibited FAK from the FERM F2 lobe by binding to the FERM F2-α3 helix, while, in CAKβ/PYK2, Ca^{2+}/calmodulin frees the kinase C-lobe in the autoinhibited CAKβ/PYK2 from the FERM F2 lobe by binding to the FERM F2-α2 helix (Figure 2). In FAK, a dimerization has not yet been shown. The crystal structure of the FAK FERM domain indicates that these two α-helices, F2-α2 and F2-α3, are immediately adjacent to the hydrophobic pocket in the FERM F2 lobe and are present at the external portion of the FERM domain, making easy access possible to these α-helices from outside by regulatory proteins. Calmodulin is the predominant intracellular receptor for Ca^{2+} with the function of a Ca^{2+} sensor. All Ca^{2+}/calmodulin-dependent protein kinases known so far are serine/threonine protein kinases. CAKβ/PYK2 is the first protein-tyrosine kinase regulated by Ca^{2+}/calmodulin.

CAKβ/PYK2 is fully tyrosine phosphorylated at its autophosphrylation site when exogenously overexpressed in cells from transfected plasmids. In this phenotype, exogenously overexpressed CAKβ/PYK2 is defferent from the endogenous protein, the tyrosine phosphorylation of which can be observed only after

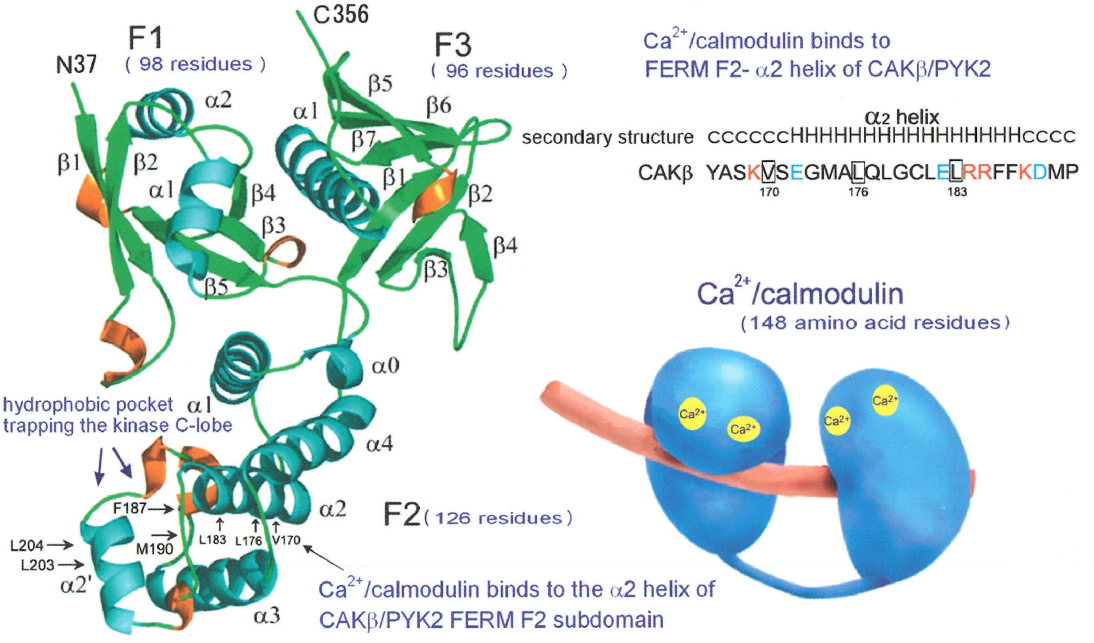

Figure 2 FERM F2-α2 helix of CAKβ/PYK2 binds Ca^{2+}/calmodulin, which presumably destroys the hydrophobic pocket trapping the kinase C-lobe under autoinhibition.

cells are stimulated with signals from outside. FAK is different from CAKβ/PYK2 in this property: exogenously overexpressed FAK is only moderately tyrosine phosphorylated. Our results indicated that exogenously overexpressed CAKβ/PYK2 was tyrosine phosphorylated by binding of Ca^{2+}/calmodulin present in cells without evident stimulation with Ca^{2+}-mobilizing extracellular ligands. 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, although Ca^{2+}/calmodulin is not involved in these cases. It has been reported that overexpressed CAKβ/PYK2 in glioblastoma is responsible for infiltrative growth of the cells. CAKβ/PYK2 regulates cell-cell repulsion and retractive cell movement induced by a spontaneous local transient increase in the intracellular free Ca^{2+}-concentration at the site of cell contacts. 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 seems to be involved in spreading and invasion.

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(Accepted for publication, Dec. 27, 2010)