A second protein-tyrosine kinase (PTK) of the focal adhesion kinase (FAK) subfamily, cell adhesion kinase β (CAKβ), was identified by cDNA cloning. The rat CAKβ is a 115.7-kDa PTK that contains N- and C-terminal domains of 418 and 330 amino acid residues besides the central kinase domain. The rat CAKβ has a homology with mouse FAK over their entire lengths except for the extreme N-terminal 88 residues and shares 45% overall sequence identity (60% identical in the catalytic domain), which indicates that CAKβ is a protein structurally related to but different from FAK. The CAKβ gene is less evenly expressed in a variety of rat organs than the FAK gene. Anti-CAKβ antibody immunoprecipitated a 113-kDa protein from rat brain, 3T3 fibroblasts, and COS-7 cells transfected with CAKβ cDNA. The tyrosine-phosphorylated state of CAKβ was not reduced on trypsinization, nor enhanced in response to plating 3T3 cells onto fibronectin. CAKβ localized to sites of cell-to-cell contact in COS-7 transfected with CAKβ cDNA, in which FAK was found at the bottom of the cells. Thus, CAKβ is a PTK possibly participating in the signal transduction regulated by cell-to-cell contacts.

Protein-tyrosine kinases (PTKs) that do not span the plasma membranes (so-called nonreceptor PTKs) have been classified into different subclasses (subfamilies) based on the sequence similarity and distinct structural characteristics (1). Many nonreceptor PTKs participate in cellular signal transduction by associating with the intracellular portions of transmembrane receptors which do not themselves have PTK activity. Different nonreceptor PTKs play diverse and specific roles in mediating the signal transduction by different nonkinase receptors (2–4).

Focal adhesion kinase (FAK) has been proposed as the prototype (and hitherto the sole member) of a new subfamily of nonreceptor PTK, represented by proteins with large N- and C-terminal domains flanking the catalytic domain but without src homology 2 and 3 (SH-2 and SH-3) domains (5–9). FAK is concentrated in focal adhesions (5, 6), and its phosphorylation and activation are triggered by the ligand binding to integrins and by the stimulation of certain growth factor and neuropeptide receptors (6, 10–24). The N- and C-terminal domains of FAK mediate its interactions with integrins, the Src-family kinases and paxillin, a focal adhesion associated protein (8, 9, 25–28). By these and other yet to be characterized interactions, FAK regulates signaling via different receptors. Because only one member of the FAK subfamily is known to date, we sought to identify a second PTK of the FAK subfamily by a homology-based cDNA cloning strategy. We describe here an isolation and characterization of a cDNA coding for a new member of the FAK family. The novel PTK described here is the second member, to our knowledge, of the FAK subfamily whose cDNA has been cloned and sequenced and is designated CAKβ for cell adhesion kinase β.

**Materials and Methods**

Amplification of PTK Catalytic Domain cDNA Fragments by PCR—PTK cDNAs were amplified from adult rat brain RNA by reverse transcriptase-directed PCR. PCR primers were designed to recognize conserved regions in PTK catalytic domains: upstream “EcoRI-FHVRDLA” primers, 5′-G-GAATTC-ATT-CTG(G/C)-CA(CT)-G(C/A)-GAT(C/T)-CT (G/T)-GC-3′; downstream “SDWSFG-BamHI” primers, 5′-TC-GGATCC-GA(T/J)-GA(T/A)-CT-CCA(G/C)-AC(G/A)-TCT-3′; where N = (A/C/G/T). RNA extracted from rat brain was reverse transcribed with the downstream primers and the Rous associated virus 2 (RAV-2) reverse transcriptase following the conditions of the manufacturer (Perkin-Elmer) in a 20-μl reaction. PCR was performed on the reverse transcriptase reaction product in a 50-μl reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 100 μg/ml gelatin, 0.2 mM dNTPs, 1.25 units of Taq polymerase (Perkin-Elmer), and 50 pmol each of the upstream and downstream primers. The thermocycling parameters used in PCR were as follows: annealing, 2 min at 55 °C; extension, 2 min at 72 °C; denaturation, 1 min at 94 °C. After 30 cycles, amplified cDNA products were digested with EcoRI and BamHI and electrophoretically separated on a 3% low melting agarose gel. An ethidium bromide-stained band at about 210 base pairs was cut out. The DNA was extracted from the gel and subcloned into pBluescript SK (+).

Nucleotide sequences were determined for 100 inserts by the dideoxynucleotide chain termination method (29) using the BcaBEST dye deoxy sequencing kit (Takara Shuzo, Otsu, Japan) and the Sequenase 2.0 kit (U. S. Biochemical Corp.), and compared with those in GenBank™ data base by the BLASTx program of NCBI (National Center for Biotechnology Information, Bethesda, MD)

**Isolation of cDNA Clones Encoding CAKβ—** A 451-bp downstream primer, M9-3, isolated from the PCR library was labeled with [α-32P]dCTP (Amersham Corp.) using a random primer labeling system (BcaBEST labeling kit, Takara Shuzo). The labeled probe was used to screen an oligo(dT)- and randomly primed adult rat brain cDNA library constructed in the λ Zap II vector (Stratagene, La Jolla, CA). Seven positive phage plaques were identified. To obtain clones covering the entire 4.0-kilobase transcript, it was necessary to rescreen the library with probes derived from the 5′- and 3′-ends of the initial cDNA isolates; 34 additional CAKβ cDNA clones were obtained by screening about 8 × 10⁶ independent clones. Nucleotide sequences were determined on both strands for selected overlap-

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Cloning and Characterization of Cell Adhesion Kinase β

Cloning and Characterization of Cell Adhesion Kinase β

Using Northern Analysis of Expression—Total RNA was extracted from the tissues of adult rat (Sprague-Dawley strain) and the indicated cell lines using ISOGEN kit (Nippon Gene, Toyama, Japan) according to the manufacturer’s protocol. RNA samples were electrophoresed through a 1.0% agarose, 2% formaldehyde gel and transferred to a nitrocellulose membrane. Hybridization to 32P-labeled fragments of the CAK β cDNA, FAK cDNA, and actin cDNA was carried out in 50% formamide, 1% SDS, 5× SSPE (1× SSPE = 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1× EDTA), 5× Denhard’s solution, 5 mM EDTA, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA at 42 °C for 14–16 h. The filters were washed (final wash: 0.2× SSC and 0.1% SDS; 1× SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.6) under conditions of either high stringency (final wash at 55 °C for 1 h) or low stringency (final wash at 43 °C for 1 h) as indicated in the figure legends. All DNA probes were radiolabeled by random priming. After hybridization, all blots were exposed to Kodak XAR film with an intensifying screen at −80 °C. DNA probes were derived from StyI fragments of the CAK β cDNA (nucleotides 74–935 and 2990–3519, which are the 5' and 3'-terminal regions). The expression of FAK was detected by hybridizing a probe derived from rat FAK cDNA (nucleotides 342–400) with mouse and human FAKs (6, 7). The rat FAK cDNA was cloned from rat brain RNA by RT-PCR using random primers designed from common amino acid sequences of FAK and CAK β. The actin probe was prepared from human β-actin cDNA.

Production of Antibodies to CAK β and Affinity Purification of the Antibody—Digestion of rat CAK β cDNA (clone 24) with Sphl and PstI restriction endonucleases generated a 688-bp base pair fragment encompassing nucleotides 2333 through 3020 of the CAK β cDNA. This fragment, encoding amino acid residues 779–1008 of CAK β, was inserted into the polylinker site of the pCAK affinity-purification vector, pSRE (38), which was derived by a modification of the original pcDNA3 vector (39). The resulting construct, pCAK β, was confirmed and sequenced. The pCAK β cDNA clone, 17N, was digested with EcoRI and subcloned into the simian virus 40-based expression vector pSR-E (38), which was derived by a modification of the original pcDNA3 vector. The resulting construct, pCAK β(S), and a control construct, pCAK β(AS), in which the CAK β cDNA was subcloned in an inverted, antisense direction, were transfected into subconfluent COS-7 cells at 10 μg/9-cm dish using DEAE-dextran (40). After 3 days, cells were harvested and lysed on ice in the lysis buffer and the lysate was analyzed by immunoprecipitation.

The epitope-tagging vector, phSV-Tag, was created by ligating a 50 mer dinucleotide (Novagen, Madison, WI) encoding the 11 amino acids (Ser-Pro-Kleu-Pro-Val-Leu). The resulting construct, pCAKβ cDNA, was transfected into subconfluent COS-7 cells at 10 μg/9-cm dish using DEAE-dextran (40). After 3 days, cells were harvested and lysed on ice in the lysis buffer and the lysate was analyzed by immunoprecipitation.

**FIG. 1.** Organization of the cloned rat CAK β cDNA. The CAK β cDNA is illustrated on a line with a position of restriction sites recognized by Aval (A), HindIII (H), KpnI (K), SacI (S), SacII (Sc), Sphl (Sp), and Xbal (X). The extended full-length 5' partial cDNA that was isolated is also shown with their names indicated to the right. M9-3 is the original PCR product. Clones 24, 119, and 114 were isolated with the PCR product as probes. Clones 17N and 21N and clones 9C, 30C, and 6C were isolated with the 5'- and 3'-portions of clone 24 as probes. The remaining clones below the CAK β line represents the predicted translation product, the CAK β protein, with the catalytic domain of the protein kinase denoted by an open box.
FIG. 2. Nucleotide sequence of rat CAKβ cDNA and deduced amino acid sequence. Nucleotides and corresponding amino acids (single-letter code) shown above are numbered at the end of each lane. The catalytic domain of the PTK is indicated by a box. The translational
stop codon is underlined. A variation of the polyadenylation signal, ATTAAA, near the 3' -end of the cDNA is also underlined. In the 5' -noncoding region, the in-frame stop codons are indicated in bold type.
acid peptide (QPELAPEDPED) derived from herpes simplex virus glycoprotein D, followed by a termination codon, into pT7Blue-T (Novagen, Madison, WI) in a sense direction. The CAKβ cDNA clone 17N was subcloned into pHSV-Tag by using a strategy that resulted in the epitope with additional N-terminal three amino acid residues, YGL, replacing the C-terminal residue, E, of CAKβ. For expression in vivo, the derivative was subcloned into pSRE to obtain pCAKβTag. The 11-residue epitope tag is specifically recognized by the anti-epitope tag monoclonal antibody.

Immune Complex Kinase Assay—CAKβ, epitope-tagged CAKβ, and FAK were immunoprecipitated from the clarified lysates (0.4 mg of protein) of COS-7 cells, transfected with cDNA constructs, and 3Y1 cells as described above. The immunoprecipitates were washed twice with 0.5 M LiCl, once with 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, and once with a kinase assay buffer (20 mM Tris-HCl (pH 7.4), 10 mM MnCl₂, 1 mM dithiothreitol) and suspended in 20 μl of the kinase assay buffer containing 5 μCi of [γ-32P]ATP (4500 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, CA). After incubation for 20 min at 20°C, the incubation was terminated by the addition of an equal volume of 2× SDS-PAGE sample buffer. A 20-μl portion of the 32P-labeled immune complexes was subjected to SDS-PAGE in a 7.5% gel. The gel was dried, and the 32P-labeled proteins were made visible by autoradiography.

For assays of protein-tyrosine kinase activity using poly(Glu,Tyr) (4:1, 20–50 kDa; Sigma), the clarified lysates (0.4 mg of protein) of transfected COS-7 cells and 3Y1 cells were incubated in 20 μl of the kinase assay buffer with 5 μg/20 μl of the exogenous substrate along with 5 μCi of [γ-32P]ATP, 5 μM unlabeled ATP, and 5 mM MgCl₂. The reactions were carried out for 10 min at 20°C, and stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer. The labeled substrate in a 20-μl portion of each assay was separated by SDS-PAGE in a 15% gel. 32P-Phosphorylated poly(Glu,Tyr) was then visualized and quantitated by bioimaging analysis of the dried gel with Bioimaging Analyzer (BAS 2000, Fuji Photo Film, Tokyo). The counts of

### Fig. 3. Comparison of nucleotide sequences of the 5'-terminal portions of rat and human CAKβ cDNAs and their deduced amino acid sequences.

The 5'-terminal portion of human CAKβ isolated from human hippocampus cDNA library was compared with the 5'-terminal portion of rat CAKβ. Dots represent gaps introduced to improve the alignment. Nucleotides and corresponding amino acids (single-letter code) shown above are numbered at the end of each lane. The in-frame stop codons are indicated in bold type.
photo-stimulated luminescence were corrected to reflect the incorporated radioactivity by subtracting the count of an appropriate control set in each series of kinase assays. Analysis of C\(\text{AK}\)/\(\beta\) Phosphotyrosine Content—To study changes in phosphotyrosine content of C\(\text{AK}\)/\(\beta\) and FAK in response to trypsinization and plating cells on fibronectin and poly-L-lysine, confluent 3Y1 cells were cultured overnight in a medium containing 0.5% fetal calf serum and harvested by trypsinization, which was stopped by adding soybean trypsin inhibitor (Sigma). The cells were suspended in serum-free Iscove’s modified Dulbecco’s medium and plated on 9-cm tissue culture dishes coated with either bovine plasma fibronectin (Biomedical Technologies, Stoughton, MA) (0.1 mg/dish) or poly-L-lysine (0.2 mg/ dish). The dishes were incubated at 37°C for 50 min, and cells attached on dishes were analyzed. Cell lysates were prepared and centrifuged at 15,000 \(\times\) g for 20 min. The detached lysates (0.6 mg protein per assay) were subjected to the immunoprecipitation with either anti-C\(\text{AK}\)/\(\beta\) beads or anti-FAK beads. After SDS-PAGE and blotting to PVDF membranes, C\(\text{AK}\)/\(\beta\), FAK, and phosphotyrosine were detected by probing blots with anti-C\(\text{AK}\)/\(\beta\), anti-FAK polyclonal antibody, and anti-phosphotyrosine, 4G10.

Confocal Laser Scanning Microscopy of Immunoconstituted COS-7 Cells—COS-7 cells, grown overnight on glass coverslips, were transfected with the indicated plasmid. After 3 days, the cells were rinsed in PBS and fixed for 10 min at 4°C in 95% ethanol. Fixed cells were preincubated with fetal calf serum for 30 min at 20°C and then incubated with primary antibodies for 1 h at 20°C. The cells were then washed three times for 10 min each in PBS. The secondary antibodies were then applied for 1 h at 20°C. After washing as above, the coverslips were mounted in PBS containing 50% glycerol and 0.02% 1,4-diazobicyclo-(2,2,2)octane (Aldrich), which was added to delay fading of immunofluorescence during microscopy. The primary antibodies used were affinity-purified anti-C\(\text{AK}\)/\(\beta\) (used at 20 \(\mu\)g of protein/ml), anti-epitope tag (used at 40 \(\mu\)g of protein/ml), and anti-FAK polyclonal antibody (used at 67 \(\mu\)g protein/ml). Secondary antibodies were fluorescein-conjugated goat anti-mouse and swine anti-rabbit immunoglobulins (Dako) Japan (used at 100-fold dilution). Immunofluorescence was imaged with a Bio-Rad MRC-500 confocal laser scanning microscope. The microscope is fitted with 60 \(\times\) (numerical aperture 1.4) objectives in connection with a Nikon Optiphot-2 upright fluorescence microscope. Digitalized fluorescence images obtained by illuminating with a 25-milliwatt multiline argon laser were filed in a 768 \(\times\) 512 pixel frame memory. A series of optical sections through each cell was taken at vertical steps of 1 \(\mu\)m. Digital image files were stored on an optomagnetic disk and were subsequently recorded on 35-mm film.

RESULTS AND DISCUSSION

Isolation of cDNA Clones Encoding a Second FAK—To identify novel members of the PTK gene family, a PCR-based approach was used. Reverse transcriptase-directed PCR was performed using RNA from adult rat brain and degenerated primers. A mixture of oligonucleotide primers coding for a PTK catalytic domain cDNA fragments. Six of these PCR products coded for already known members of receptor PTKs. A computer-assisted sequence analysis of one other PCR fragment, M9-3, showed that it encoded a novel amino acid sequence with the conserved residues characteristic of PTKs. Among the known PTKs, the highest homology was found with the PTK catalytic domain of FAK (76% identity in the translated amino acid sequence). M9-3 is clearly related to but distinct from the cDNA of the mouse, human, and chicken FAKs (5-7); it was evident that M9-3 does not code for a rat homologue of FAK. M9-3, a 201-base cDNA fragment, was subsequently used as a probe for screening a commercially available rat brain cDNA library made by random and oligodeoxythymidylic acid (oligo(dT)) primers. The screening of about 6 \(\times\) 10\(^5\) independent clones allowed us to obtain seven overlapping cDNAs covering the major portion of the mRNA (Fig. 1). The cDNA clones covering 5'- and 3'-terminal regions of the mRNA were obtained by screening the library with the 5'- and 3'-portions of the initial overlapping cDNAs as probes. We present in Fig. 2 a composite sequence of 4.05 kilobases deduced from these cDNA fragments, together with the sequence of the protein, which we call C\(\text{AK}\)/\(\beta\).

C\(\text{AK}\)/\(\beta\) is a New Member of the FAK Subfamily of PTKs—The combined 4048-base pair cDNA contained a long open reading frame encoding a protein of 1009 amino acid residues with calculated molecular mass of 115,724 Da, which has all the characteristics of a nonreceptor PTK. The open reading frame is flanked by a 5'-untranslated sequence of 261 base pairs and a 3'-untranslated sequence of 757 base pairs. The 3'-extremity of the cDNA contains the polyadenylation signal 5'-ATTAAA-3', followed closely by 12 consecutive terminal adenine residues. The proposed initiation codon at nucleotides 1–3 is of the form 5'-GAGAGATTCC-3', which represents a suboptimal primary sequence context for initiation of translation with a purine at position -3, but without a G at position +4 (41). The assignment of ATG at nucleotides 1–3 as the initiation codon is confirmed by the 5'-terminal sequence analysis of a human C\(\text{AK}\)/\(\beta\) cDNA, which we cloned from human hippocampus cDNA library. The nucleotide sequences are well conserved in the putative coding regions of rat and human C\(\text{AK}\)/\(\beta\) cDNAs, resulting in the almost identical N-terminal amino acid sequences of rat and human C\(\text{AK}\)/\(\beta\) (Figs. 3 and 4). The 5'-noncoding regions of rat and human CAK/\(\beta\) cDNAs contain an in-frame TAG stop codon at positions 18–20 and 16 and the rat and human cDNA sequences clearly diverge from the positions of about 60 to the 5'-extremity (Figs. 3 and 4).

A protein kinase catalytic domain typical of the PTKs (1) and including the sequence identical to that encoded by the M9-3 PCR fragment encompasses amino acids 419–679 (Fig. 2). The deduced protein contains a 418 amino acid N-terminal and a 330 amino acid C-terminal noncatalytic domains. A comparison of the C\(\text{AK}\)/\(\beta\) cDNA sequence by the BLASTx program of NCBI with those in GenBankTM data base revealed homology of C\(\text{AK}\)/\(\beta\) and FAK over their entire lengths, and did not detect any sequence more closely related to C\(\text{AK}\)/\(\beta\). Comparison of the deduced amino acid sequence of the encoded protein with those of mouse, human, and chicken FAKs revealed that this cDNA
FIG. 5. Comparison of amino acid sequences of rat CAKβ and mouse FAK. The numbers on the right indicate the positions relative to the putative start methionine of CAKβ and the amino acid residue number of mouse FAK (6). The catalytic domains are boxed. Amino acid residues of FAK identical with those of CAKβ are indicated by dashes. Dots represent gaps introduced to improve the alignment. The sequences of FAK at residues 861–882, 711–741, and 684–705 are duplicated at the sequences of CAKβ where local homology are found with the duplicated FAK sequences. The tyrosine phosphorylation site discussed in the text is underlined.
Fig. 6. Comparison of the PTK catalytic domain of CAKβ with other PTKs. Identical residues are indicated by dashes. Dots represent gaps introduced to improve the alignment. Asterisks denote amino acid residues that are highly conserved among PTKs (1) and appear in CAKβ. Residues which are highly conserved among PTKs, but different in CAKβ, are indicated by the symbol, #. The number in parentheses is the percentage of residues that are identical with CAKβ. PTK sequence data were taken from the GenBank™ database and the accession numbers are given in parentheses; mouse FAK (M95408), human FAK (L13616), rat Flik (X13412), mouse ZAP-70 (U04379), mouse EGFR (X78987), human FER (J03358), human Arg (M35296), mouse Tec (X55663), rat Hck (M83666), chicken c-Src (V00402).
encoded a FAK-related but distinct PTK (Fig. 5). The amino acid sequence and the structural organization of CAKβ clearly indicate that CAKβ is a PTK of the FAK subfamily. The unique overall architecture of FAK that the catalytic domain is flanked by large N-terminal and C-terminal domains (5, 9) is also found in CAKβ. The amino acid sequence of the catalytic domain of CAKβ is 60% identical with the catalytic domains of mouse and human FAKs (Fig. 6). The amino acid sequences of the N- and C-terminal domains of CAKβ are 39 and 40% identical with those of mouse FAK (Fig. 5). As in FAK, CAKβ contains neither SH-2 nor SH-3 domains. FAK are highly conserved evolutionarily between species; human FAK shares 97% amino acid identity with mouse FAK and 95% identity with chicken FAK (7). The result that rat CAKβ shares only 45% amino acid identity with mouse FAK indicates that CAKβ is the second PTK of the FAK subfamily. Indeed, we have amplified rat FAK in addition to CAKβ from rat brain RNA by RT/PCR using degenerated oligonucleotide primers designed from the amino acid sequences common to both FAK and CAKβ.

Homology of CAKβ and FAK—The predicted amino acid sequence of CAKβ contains isoleucine in one of the PTK-specific peptide sequences, Asp543–Ile-Ala–Val–Arg–Asn548 (Figs. 5 and 6). The isoleucine at residue 550 is characteristic to FAK (5); valine is found at the analogous position in some other PTKs. The valine at residue 552 is unusual since alanine is found at the analogous position in most of the other PTKs including FAK (6), with exceptions of several PTKs, which contain threonine or serine. CAKβ contains other PTK-specific peptide sequences, Pro588–Ile–Lys–Trp–Met592 and Ser600–Asp–Val–Trp609, and the structural motifs conserved in all protein kinases (1) including an ATP-binding site, three residues predicted to interact with the γ-phosphate group of the bound ATP, and the catalytic site Asp549. In addition to the replacement of conserved leucine by isoleucine at the residue 550 of CAKβ, three other residues highly conserved in the other PTK catalytic domains are not conserved in CAKβ (Fig. 6). Two of these at the residues 536 and 626 of CAKβ are not conserved in FAK as well. The other one at the residue 612 of CAKβ is alanine. The corresponding residue in FAK is glycine, the conserved amino acid of this position in the PTK catalytic domain. Conversely, Met537 of CAKβ is the residue highly conserved in the PTK catalytic domains but is replaced to leucine in FAK (Fig. 6).

Comparisons of the N- and C-terminal nonkinase domains between CAKβ and FAK are shown in Fig. 5. Although the amino acid residues 89–418 of CAKβ are highly homologous...
FAK, the sequence of the extreme N-terminal 88 residues of FAK, the tyrosine 397 at the juncture of the N-terminal and catalytic domains is the site of autophosphorylation and is the major in vivo and in vitro site of tyrosine phosphorylation (28). This phosphorylated tyrosine and the sequence around it are the binding site for a SH-2 domain of the Src family PTKs to FAK (27). The sequence around the Tyr397 is Glu-Thr-Asp-

(47.6% identity) with the corresponding N-terminal domain of FAK, the sequence of the extreme N-terminal 88 residues of CAKβ is entirely different from any portion of FAK (Fig. 5). This difference may imply specific binding of CAKβ to the cytoplasmic domain of some receptors other than integrins. The binding site of FAK to integrins has been identified in the cytoplasmic domain of some receptor other than integrins. The binding site for a SH-2 domain of the Src family PTKs to FAK may possibly function as ligands to the SH-3 domains of some proteins involved in the signal transduction. There is also a proline-rich stretch in the extreme N-terminal region of CAKβ (residues 18-30).

The residues 869–999 of CAKβ continuous with the C-terminal end of the proline-rich cluster are highly homologous (61.83% identity) with the residues 913-1043 of mouse FAK (Fig. 5). The region targeting FAK to focal adhesion was located to reside within the 159 residues of chicken FAK between amino acid positions 853 and 1012, which correspond residues 851-1011 of mouse FAK (26). Thus the sequence of CAKβ

\[ \text{Asp-Tyr}^{397}\text{-Ala-Glu-Ile in chicken, mouse, and human FAKs (5-7). A homologous sequence, Glu-Ser-Asp-Ile-Tyr}^{402}\text{-Ala-Glu-Ile, is found in CAKβ at the juncture of the N-terminal and catalytic domains (Figs. 2 and 5). The sequence, Tyr-Ala-Glu-Ile, common to both FAK (Tyr}^{397}\text{) and CAKβ (Tyr}^{402}\text{) conforms to a consensus high affinity binding site for the SH-2 domains of the Src family of PTKs (42).}

The sequence of the C-terminal domain, a region following the kinase domain, is 46 amino acids shorter in CAKβ as compared with FAK. In the sequence comparison presented in Fig. 5, three gaps were introduced in the C-terminal domain of CAKβ to maximize the homology with FAK. As shown in Fig. 5, the C-terminal domain of CAKβ immediately after the PTK catalytic domain (residues 699–720, 747–777, and 778–799) has local homologies with three C-terminal domain stretches of the FAK sequence (residues 861–882, 711–741, and 684–705) in a reverse order; more C-terminal sequences of FAK are homologous with more N-terminal sequences of CAKβ. It should be noted that the residues 711–741 and 861–882 of FAK are the two most proline-rich stretches in the FAK sequence (5-7). The C-terminal nonkinase region of CAKβ contains two proline-rich stretches, residues 701–767 and 831–869, where the proline content exceeds 20%. The presence of proline-rich stretches has been recognized as a characteristic element of the FAK C-terminal domain (5). The proline-rich stretches of CAKβ may possibly function as ligands to the SH-3 domains of some proteins involved in the signal transduction. There is also a proline-rich stretch in the extreme N-terminal region of CAKβ (residues 18–30).

The residues 869–999 of CAKβ continuous with the C-terminal end of the proline-rich cluster are highly homologous (61.83% identity) with the residues 913-1043 of mouse FAK (Fig. 5). The region targeting FAK to focal adhesion was located to reside within the 159 residues of chicken FAK between amino acid positions 853 and 1012, which correspond residues 851-1011 of mouse FAK (26). Thus the sequence of CAKβ
immune complex kinase assays with poly(Glu,Tyr) as a substrate on lysates of COS-7 cells, transfected with CAK β-cDNA constructs, and 3Y1 cells

CAK β was immunoprecipitated with the indicated antibody beads from the lysates of COS-7 cells transfected with pCAK β(S), pCAK β(AS), or pCAK β(TAG), and from the 3Y1 cell lysate. FAK was immunoprecipitated with anti-FAK beads from the 3Y1 cell lysate. Protein-tyrosine kinase activity was assayed with poly(Glu,Tyr) as an exogenous substrate as described under "Materials and Methods." S, sense; AS, antisense.

| Series | Cells | Plasmids | i.p. | PSL |
|--------|-------|----------|------|-----|
| I      | COS-7 | pCAK β(AS) | αCAK β | 0   |
|        | COS-7 | pCAK β(S)  | αCAK β | 5,297 |
|        | COS-7 | pCAK βTag | αCAK β | 6,202 |
| II     | COS-7 | pCAK β(S)  | αTag | 12,438 |
|        | COS-7 | pCAK βTag | αTag | 6,600 |
| III    | 3Y1   | pre | 0   |
|        | 3Y1   | αCAK β | 6,127 |
|        | 3Y1   | αFAK | 6,127 |

a i.p., immunoprecipitation.
b PSL, counts of photo-stimulated luminescence.
c αCAK β, anti-CAK β beads; αTag, anti-epitope tag beads; pre, pre-immune serum beads; αFAK, anti-FAK beads.

Table I

Fig. 12. Analysis of phosphorylation of CAK β and FAK in 3Y1 cells before and after trypsinization and 50 min after plating the cells onto fibronectin or poly-L-lysine. The 3Y1 cell lysates were prepared by adding the lysis buffer either directly to the washed cells on dishes (lanes 1, 3–5, 7, and 8) or to the trypsinized cells (lanes 2 and 6) (Off Dish). The lysates of cells on dishes were prepared either from cells before trypsinization (On Dish), from cells 50 min after plating on fibronectin, or from cells 50 min after plating on poly-L-lysine. A, immunoprecipitation with anti-CAK β. Immunoblotting was either with anti-CAK β (lanes 1–4) or with anti-phosphotyrosine (lanes 5–8). B, immunoprecipitation with anti-FAK. Immunoblotting was either with anti-FAK (lanes 1–4) or with anti-phosphotyrosine (lanes 5–8).

Between positions 845 and 967 may possibly contain the target sequence of CAK β to a certain submembranous site. On the other hand, the CAK β sequence of the extreme C-terminal 10 amino acids, residues 1000–1009, is not homologous with the C terminus of FAK. It has been reported that a replacement of the extreme C-terminal 13 residues of FAK with an epitope tag blocks paxillin binding to FAK (8). Therefore, CAK β may bind not to paxillin but to some other proteins associated with the cytoplasmic side of the surface membrane.

Expression of the CAK β Gene Transcripts—We have searched for CAK β gene expression in rat tissues by hybridization of the cDNA fragments, 5′-coding region and 3′-coding/noncoding regions of the CAK β cDNA, to a Northern blot carrying RNA from the following adult rat tissues: whole brain without cerebellum, cerebellum, lung, liver, kidney, spleen, intestine, testis, epididymis, adrenal gland, pancreas, and skeletal muscle. The Northern blots were also probed with a rat FAK probe as a reference. Transcripts of about 4.4 kilobases, almost the same size as FAK mRNA, were detected in whole brain, intestine, kidney, spleen and epididymis (Figs. 7 and 8). The same results were obtained with the CAK β 5′- and 3′- cDNA probes. CAK β mRNA is particularly abundant in whole brain without cerebellum. The transcripts are scanty in cerebellum, testis, and adrenal gland; in these organs the FAK gene transcripts are abundant (Fig. 7). The 4.4-kilobase transcripts were also detected in rat fibroblast lines, WFB and 3Y1 (Fig. 8). These cell lines also express mRNA for FAK (Fig. 8). In a human T cell leukemia line, Jurkat, transcripts of 4.6 kilobases were detected with both CAK β and FAK probes (Fig. 8). No significant CAK β gene transcript was found in mouse fibroblast lines (Fig. 8), BALB/3T3, Swiss/3T3, or NIH/3T3, a monkey cell line, COS-7 (Fig. 8), or rat and mouse neural cell lines (data not shown), PC12, NIE115, and NG108-15. In 3T3 lines and COS-7, the expression of the FAK gene was confirmed (Fig. 8).

Detection of CAK β in Rat Brain and 3Y1 Cells—Anti-CAK β antiserum was raised by immunizing rabbits with a bacterially expressed TrpE fusion protein containing the extreme C-terminal 230 amino acids of CAK β (amino acid residues 779–1008). Anti-CAK β was affinity-purified on a column of a covalently bound glutathione S-transferase fusion protein of the CAK β C-domain to Sepharose. Anti-CAK β specifically immunoprecipitated and immunoblotted a protein of about 113-kDa (equivalent to the calculated mass of CAK β) from the lysates of rat brain, 3Y1 cells and SR-3Y1 cells, a src-transformed line of 3Y1 (Fig. 9, lanes 2, 4, and 5). In accordance with the calculated molecular masses, the immunologically identified CAK β has a faster mobility in SDS-PAGE than FAK, which was immunoprecipitated from the 3Y1 cell lysate with anti-FAK monoclonal antibody, 2A7 (36), and immunoblotted with polyclonal anti-FAK antibody (Fig. 9, lane 9). Immunoblotting with anti-phosphotyrosine revealed a band at CAK β on the blotted membrane from a SDS-PAGE gel where the anti-CAK β immunoprecipitates from the lysates of rat brain, 3Y1 cells and SR-3Y1 cells were separated (Fig. 9, lanes 6–8). CAK β of SR-3Y1 cells was stained more strongly with anti-phosphotyrosine than CAK β of 3Y1 cells, indicating higher in vivo tyrosine-phosphorylation of CAK β in the src-transformed cells; compare the CAK β band density in lane 7 divided by that in lane 4 with that in lane 8 divided by that in lane 5.

CAK β cDNA Encodes pp113CAK β—The pCAK β(S)-transfected COS-7 cells but not the pCAK β(AS), an antisense construct, transfected COS-7 cells or the mock-transfected COS-7 cells expressed CAK β of the same size as was detected in 3Y1 cells (Fig. 10A, lanes 1–5). Immunoprecipitation and immunoblotting with anti-CAK β was used to detect the expression. The CAK β expressed in COS-7 cells was also tyrosine-phosphorylated (Fig. 10A, lane 7). An epitope-tagged CAK β was expressed in the COS-7 cells transfected with pCAK βTag and contained an 11-amino acid epitope tag plus 3 amino acid residues in place of the C-terminal glutamic acid. The tagged CAK β was immunoprecipitated (Fig. 10B, lanes 4 and 8) and immunoblotted with anti-epitope tag monodonal antibody (Fig. 10B, lanes 7 and 8). The tagged CAK β was also immunoprecipitated (Fig. 10B, lanes 3 and 7) and immunoblotted with anti-CAK β (Fig. 10B, lanes 3 and 4). The anti-epitope tag did not bind CAK β itself (Fig. 10B, lanes 2, 5, and 6).

In Vitro Phosphorylation of CAK β in Immune Complex Assays and Demonstration of PTK Activity—Immune complexes formed by incubating cell lysates with anti-CAK β and anti-epitope tag were assayed for protein kinase activity with [γ-32P]ATP as the phosphate donor without adding exogenous acceptor, and the 32P-labeled immune complexes were analyzed by SDS-PAGE. A protein of about 113-kDa, the size of CAK β , was found to become 32P-phosphorylated (Fig. 11). The
$\text{32P}^\text{labeling of the protein was found in the kinase assays of the immunoprecipitate from the 3Y1 cell lysate with anti-CAK}\beta$ (Fig. 11, lane 8), of that with anti-CAK$\beta$ from the pCAK$\beta$(S)-transfected COS-7 cell lysate (Fig. 11, lane 3), of that with anti-CAK$\beta$ from the pCAK$\beta$(S)Tag-transfected COS-7 cell lysate (Fig. 11, lane 5), and of that with anti-epitope tag from the pCAK$\beta$(S)Tag-transfected COS-7 cell lysate (Fig. 11, lane 6). The $\text{32P}$ labeling of the 113-kDa protein was not found in the kinase assays of the control immunoprecipitate with anti-CAK$\beta$ from the pCAK$\beta$(AS)-transfected COS-7 cell lysate (Fig. 11, lane 1), of that with anti-epitope tag from the pCAK$\beta$(S)-transfected COS-7 cell lysate (Fig. 11, lane 4), or of the immunoprecipitates prepared by preimmune serum (Fig. 11, lanes 2 and 7). When an immunoprecipitate with anti-FAK from the 3Y1 cell lysate was subjected to the in vitro kinase assay, a 125-kDa protein was $\text{32P}$-phosphorylated and tentatively identified as the autophosphorylated FAK (Fig. 11, lane 9). These results indicate that the 113-kDa protein revealed by the in

**Fig. 13.** Immunostaining of CAK$\beta$ in the pCAK$\beta$Tag-transfected COS-7 cells with anti-epitope tag viewed with optical sections obtained by confocal microscopy. The pCAK$\beta$Tag-transfected COS-7 cells (A and B) and the pCAK$\beta$-transfected COS-7 cells, a control (C and D), were immunostained with anti-epitope tag. Images of optical sections at 0 $\mu$m (A and C) and 4 $\mu$m (B and D) above the surface of coverslips were obtained with a confocal laser scanning microscope. The calibration bar in C represents 10 $\mu$m.

**Fig. 14.** Immunostaining of CAK$\beta$ in the pCAK$\beta$Tag-transfected COS-7 cells with anti-CAK$\beta$ viewed with optical sections obtained by confocal microscopy. The pCAK$\beta$Tag-transfected COS-7 cells (A and B) and the mock-transfected COS-7 cells, a control (C and D), were immunostained with anti-CAK$\beta$. Images of optical sections at 0 $\mu$m (A and C) and 4 $\mu$m (B and D) above the surface of coverslips were obtained with a confocal laser scanning microscope. The calibration bar in C represents 10 $\mu$m.
vitro $^{32}$P-phosphorylation is the CAKβ autophosphorylated in vitro.

To confirm the PTK activity of CAKβ, the immune complexes formed with anti-CAKβ and anti-epitope tag were assayed for the kinase activity with poly(Glu,Tyr) as an exogenous substrate. CAKβ and the tagged CAKβ immunoprecipitated from the transfected-COS-7 cells efficiently catalyzed the phosphorylation of the substrate (Table I). CAKβ and FAK immunoprecipitated from 3Y1 cells also catalyzed the phosphorylation (Table I).

Tyrosine phosphorylation of CAKβ is not enhanced in response to plating 3Y1 Cells onto Fibronectin—as has been shown in 3T3 cells (6, 43), the phosphorysotryne content of FAK decreased on detachment of 3Y1 cells by trypsinization (Fig. 12B, lanes 5 and 6) and regained on plating the cells onto fibronectin but not on plating onto poly-L-lysine (Fig. 12B, lanes 5, 7, and 8). To test the possible interaction of CAKβ with integrin and fibronectin, we investigated the effects of trypsinization and plating of 3Y1 cells on fibronectin-coated dishes on the tyrosine-phosphorylated state of CAKβ by immunoblotting with anti-phosphotyrosine. The tyrosine-phosphorylated state of CAKβ was not affected by trypsinization (Fig. 12A, lanes 5 and 6) or by plating the cells onto fibronectin or poly-L-lysine (Fig. 12A, lanes 5, 7, and 8). A control blot probed with anti-CAKβ verified that equal amounts of CAKβ were present in the lysates (Fig. 12A, lanes 1–4). The results indicate that CAKβ is not activated in response to cell interactions with fibronectin, suggesting the association of CAKβ with a cell surface molecule other than integrin.

CAKβ localizes to sites of cell-to-cell contact but not to sites of focal adhesion—Anti-epitope tag antibody and affinity-purified anti-CAKβ antibodies were used to localize CAKβ in the pCAKβ Tag-transfected COS-7 cells by immunostaining. Confocal laser scanning microscopy made it possible to locate FAK at the base of the cells and CAKβ at the cell-to-cell contact (Figs. 13–15). As shown in Fig. 8, COS-7 cells express FAK mRNA but not CAKβ mRNA. Therefore, the endogenous FAK and CAKβ expressed from the transfected pCAKβ Tag were examined by the immunostaining. In the confluent pCAKβ Tag-transfected cells, both anti-epitope tag and anti-CAKβ stained the sites of cell-to-cell contact at the middle to upper portions of the cells (Figs. 13B and 14B). The confluent cells were about 6 μm tall, and the images of optical sections 4 μm above the base of the cells were presented in Figs. 13B and 14B to show the pericellular stainings. Such pericellular staining was not found in the pCAKβ-transfected cells, a control, on the anti-tag staining (Fig. 13D), or in the mock-transfected cells on the anti-CAKβ staining (Fig. 14D). The nuclear immunofluorescence was not specific to the pCAKβ Tag-transfected cells but was also found in the control cells (Figs. 13B and D, and 14B and D), and thus considered to be mostly nonspecific. FAK was immunolocalized at the bottom of COS-7 cells in a patchy distribution around the base of nucleus (Fig. 15A). The sites of immunostained FAK probably represent focal adhesions of the cells. The presence of focal adhesions at the bottom of COS-7 cells was confirmed by confocal interference reflection microscopy (44) (data not shown). Neither significant staining at the cell bottom with anti-epitope tag (Fig. 13, A and C) and with anti-CAKβ (Fig. 14A and C) nor significant staining with anti-FAK at the middle of the cells, optical sections at 4 μm, (Fig. 15B) was found in images of these optical sections.

One of the most important questions on CAKβ to be answered is the identification of the cell adhesion molecule, which associates with CAKβ, and activates and tyrosine-phosphorylates CAKβ. Localization of CAKβ to the sites of cell-to-cell contact and not to focal adhesions suggests an association of CAKβ with some cell-to-cell adhesion molecule. Thus, FAK is the first cell adhesion kinase participating in the signal transduction triggered by cell interactions with the extracellular matrix, while CAKβ is the second cell adhesion kinase with a possibility to participate in the signal transduction pathway regulated by cell-to-cell adhesions.

The finding that WFB and J urkat express CAKβ as well as FAK raises an interesting question: whether any receptor known to be present in these cells is coupled to the activation and phosphorylation of CAKβ. WFB responds to vasopressin, endothelin, bombesin, prostaglandin F2α, and PDGF with the activation of phospholipase C and an elevation of intracellular free Ca$^{2+}$ concentration (45). In J urkat cells, tyrosine kinase pathways are activated by the ligation of the T cell receptor complex (46). Recently, Kanner et al. (47) reported that ligation of the antigen receptors on T and B lymphocytes rapidly augments the tyrosine phosphorylation of a FAK-related protein, which they denote fakB. The human FAK peptide sequence, against which they raised fakB-reactive antisemur, 714, is only 3 out of 20 residues identical with the corresponding sequence, residues 683–701, of rat CAKβ. Therefore, fakB is not likely to be CAKβ.

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