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Restricted Expression of Cell Adhesion Kinase-β in Rat Tissues

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Cell adhesion kinase-β (CAK-β) is a protein tyrosine kinase of the focal adhesion kinase subfamily, which contains large amino- and carboxyl-terminal domains. We studied the tissue distribution of CAK-β and its mRNA by immunohistochemical staining and* in situ *hybridization. In rat brain, CAK-β was mainly found in the medulla whereas CAK-β mRNA was expressed in most neurons, especially pyramidal cells and Purkinje cells. In the small intestine, CAK-β protein and mRNA were detected in the absorptive epithelial cells, and the protein was concentrated in the brush border. Double immunostaining for CAK-β and actin showed that they co-localized in the brush border of small intestine cells. Immunoelectron micrography revealed that the anti-CAK-β antibody localized within microvilli. In the kidney, the protein was mainly expressed in proximal tubular cells, which have well developed microvilli, although CAK-β mRNA was observed in most urinary tubular cells. In other tissues, the ciliated cells of the epididymis strongly expressed CAK-β mRNA and CAK-β localized in the cilia. In addition, α- and β-tubulin were identified in the rat brain lysates immunoprecipitated with anti-CAK-β antibody. The present results demonstrate that CAK-β is present at relatively high levels in cilia, axons, and microvilli. This suggests that CAK-β may play important roles in the functions of these structures or that the CAK-β-related signaling pathway is closely associated with cytoskeletal components. (Am J Pathol 1997, 150:267–281)

The activation of protein tyrosine kinases (PTKs) is one of the most common signal transduction mechanisms directly coupled to receptor activation by external signals. Most of the transmembrane receptors for protein growth factors are by themselves tyrosine kinases, receptor-PTKs. The PTKs that do not span the plasma membrane (the so-called nonreceptor PTKs) have been classified into different subclasses (subfamilies) based on sequence similarities and distinct structural characteristics.[1] We found, by cDNA cloning, a new nonreceptor PTK, which we named cell adhesion kinase-β (CAK-β).[2] CAK-β is the second PTK of the focal adhesion kinase (FAK) subfamily. CAK-β has an amino-terminal domain of 418 amino acid residues and a carboxyl-terminal domain of 330 amino acid residues in addition to the 261 amino acid residues of the central kinase domain but without Src homology 2 and 3 (SH-2 and SH-3) domains. The cDNAs of the protein have been cloned from rat,[2] mouse,[3] and human.[2–6] The amino acid sequence of human CAK-β is 95.4% identical with that of rat CAK-β.[6] Human CAK-β and the human FAK share sequence identities of 39, 60, and 39% at their amino-terminal, kinase, and carboxyl-terminal domains, respectively. CAK-β has been named PYK2,[4] RAFTK,[3] and FAK2[5] by other groups of researchers. The expression of CAK-β mRNA is high in brain, intestine, kidney, spleen, and lung,[2] although the CAK-β gene is less evenly expressed than the FAK gene in a variety of organs.

The cell physiological function of CAK-β remains mostly unknown. In general, nonreceptor PTKs participate in signal transduction by associating with intracellular portions of the transmembrane receptors that do not themselves have PTK activity. It has been shown that there are specific associations between the nonreceptor PTKs of a subfamily and the nonkinase receptors of a specific group, resulting in transduction through the associated PTKs of the sig-

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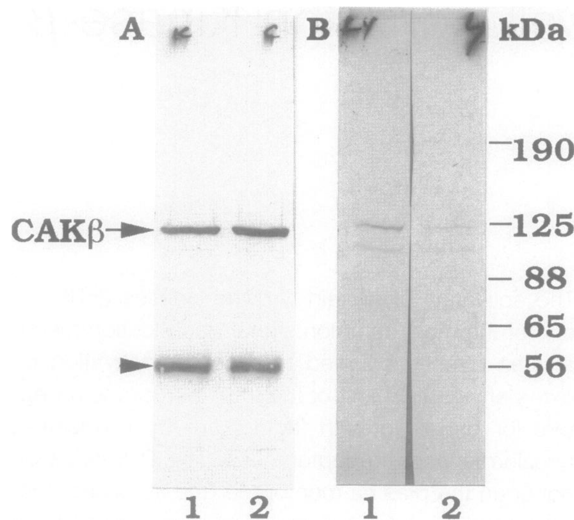

Figure 1. *Specificity of the anti-CAK-β rabbit antibodies.* **A:** *Immunoprecipitation of rat brain lysate with affinity-purified anti-CAK-β rabbit antibodies directed to residues 670 to 716 of CAK-β (anti-CAK-β (C-a);* **lane 1**) *and to residues 779 to 1008 of CAK-β (anti-CAK-β (C-b);* **lane 2**). *The blotted membrane was probed with the antibody against residues 670 to 716 of CAK-β. The band at 55 kd* (**arrowhead**) *was also found when protein A-Sepharose without bound anti-CAK-β was mixed with the brain lysate and analyzed.* **B:** *A total lysate of the rat brain (30 μg of protein) was subjected to electrophoresis and blotted to a PVDF membrane. The blot was probed with anti-CAK-β (C-a) antibodies* (**lane 1**) *and with anti-CAK-β (C-b)* (**lane 2**).

nals received by the receptors. For example, Syk and ZAP70 participate in signaling through immune recognition receptors, and JAK family PTKs participate in signaling through cytokine receptors.[7,8] FAK mediates signaling through integrins; a complex assembly of proteins is formed in association with FAK, which results in the activation of several signaling pathways.[9,10] FAK is also activated by stimulation of receptors coupled to phospholipase C activation such as neuropeptide receptors and the platelet-derived growth factor receptor.[11] This second mode of activation is also found in CAK-β. It has been shown that the tyrosine phosphorylation of CAK-β is markedly enhanced when the cytoplasmic free Ca^{2+} concentration is increased.[4] However, the cell surface receptor that may possibly associate with CAK-β, just as integrin does with FAK, remains to be identified. Thus, the signaling cascade by which CAK-β is activated mostly remains to be elucidated.

CAK-β has at least three characteristic ligand sequences in common with FAK. The first one is the autophosphorylation site (residues 402 to 405), and it participates in binding to the SH-2 domains of the Src family kinases.[12] The second one is the ligand sequence to the SH-3 domains of $pp130^{cas}$ and Efs (residues 712 to 719).[13,14] The third one works as the ligand to the SH-2 domain of Grb2 (residues 881 to 884) when phosphorylated.[15] These and other structural and functional characteristics common to both CAK-β and FAK indicate that CAK-β has an important function as a docking protein in activating diverse intracellular signaling pathways at the site of some unidentified cell structure with which CAK-β associates. The extreme amino-terminal 88 amino acid residues of CAK-β are entirely different from FAK and unique to CAK-β, raising the possibility that this region is involved in a specific function of CAK-β different from FAK.

As a part of the study to elucidate the cell physiological function of CAK-β, we studied the tissue distribution of CAK-β by immunohistochemical staining of CAK-β and by *in situ* hybridization. The results obtained in this study indicate that CAK-β is not present in all cells or evenly in a group of cells but is concentrated in specific cell structures of several differentiated cells such as nerve axons, brush borders of small intestinal epithelial cells and of renal proximal tubular cells, and cilia. Furthermore, tubulins were co-immunoprecipitated with CAK-β by anti-CAK-β from the rat brain lysate. The results suggest that CAK-β is intimately associated with the functions of these cell structures by binding to cytoskeletal proteins.

Materials and Methods

Adult Sprague-Dawley rats were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). All animals had free access to food and water. Commercial sources of antibodies were as follows: mouse monoclonal anti-FAK antibody, Transduction Laboratories (Lexington, KY); mouse monoclonal anti-α-tubulin and anti-β-tubulin antibodies, Amersham International (Little Chalfont, UK); rabbit anti-actin antibody, ICN ImmunoBiologicals (Lisle, IL); rabbit anti-α-catenin antibody, monoclonal anti-rabbit immunoglobulins (clone RG-16) conjugated with alkaline phosphatase, goat anti-mouse IgG conjugated with alkaline phosphatase, and rhodamine-conjugated phalloidin, Sigma Chemical Co. (St. Louis, MO); peroxidase-conjugated goat anti-rabbit IgG, biotinylated goat anti-rabbit IgG, biotinylated rabbit anti-mouse IgG, and Vectastain ABC Elite kit, Vector Laboratories (Burlingame, CA); fluorescein-isothiocyanate-conjugated anti-rabbit Ig and rhodamine-conjugated anti-mouse Ig antibodies, Dako (Glostrup, Denmark); and gold-conjugated anti-digoxigenin antibody, Boehringer Mannheim (Mannheim, Germany). APS-coated glass slides were obtained from Matsunami (Tokyo, Japan). Block Ace was purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). The digoxigenin RNA labeling kit, T3 and T7

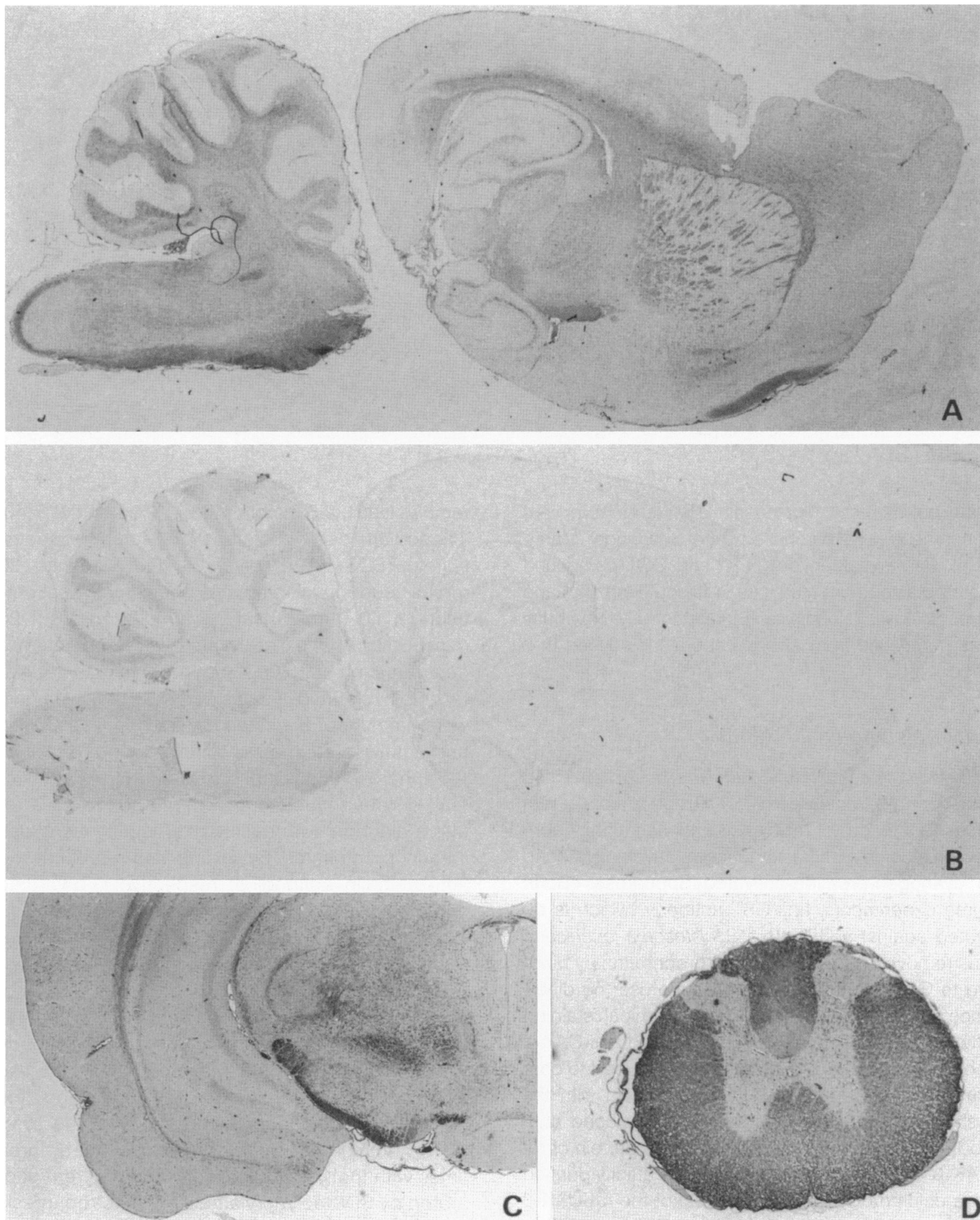

Figure 2. *Immunohistochemical staining of adult rat brain with anti-CAK-β (C-a) antibody.* **A:** *Sagittal section. Positive staining is present mainly in the neural tracts such as the corpus callosum, optic tract, geniculate bodies, cerebral peduncle, cerebellar medulla, and pyramidal tracts. Magnification, ×9.* **B:** *Control section showing specificity of the immunostaining. The section was processed as in* **A**, *except that the primary antibody was omitted. Magnification, ×9.* **C:** *Coronal section. Positive staining is mainly found in the corpus callosum, thalamus, and cerebral peduncle. Magnification, ×12.5.* **D:** *Spinal cord. Strongly positive immunostaining with anti-CAK-β is found in the white matter. Magnification, ×16.5.* **E:** *Cerebral cortex. Pyramidal cells are positive in the immunostaining. Magnification, ×165.* **F:** *Cerebellum. Purkinje cells and cerebellar tracts are positively stained. The granular cells are negative. Magnification, ×165. No counterstaining was done.*

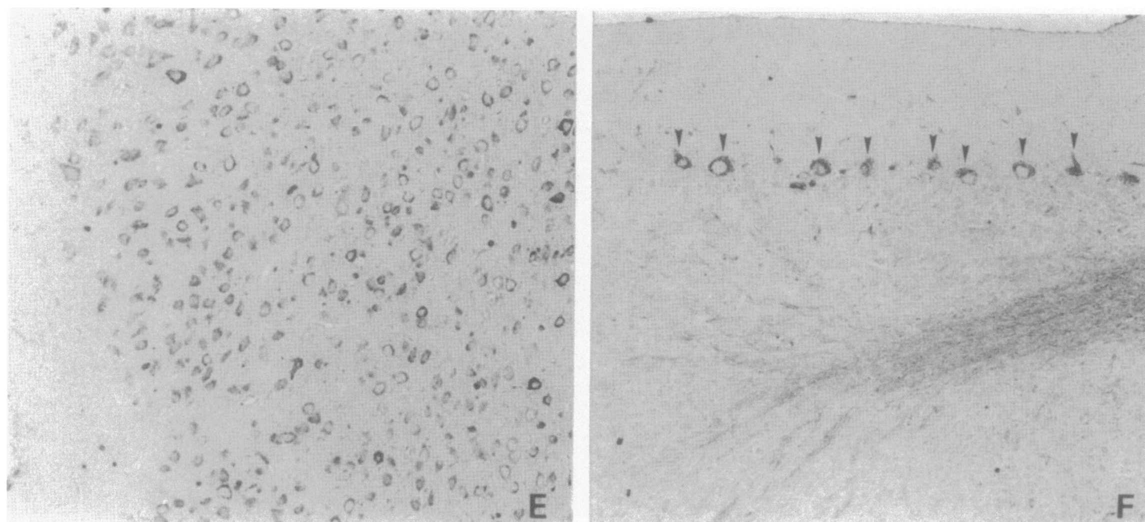

Figure 2. *Continued.*

RNA polymerase, digoxigenin-UTP, and silver enhancement were purchased from Boehringer Mannheim. Paraformaldehyde (PFA) and OCT compound were obtained from Merck (Darmstadt, Germany) and Miles Scientific (Naperville, IL), respectively. Nembutal was purchased from Abbott Laboratories (North Chicago, IL).

Antibodies against CAK-β

The antibody used in this study was raised against a glutathione S-transferase (GST) fusion protein of residues 670 to 716 of rat CAK-β and was affinity purified on a column of the immunogen covalently bound to cyanogen-bromide-activated Sepharose 4B. In some experiments, anti-GST antibody, which is directed against a GST from *Schistosoma japonicum*, was removed from the antibody preparation by binding to GST-Sepharose beads before use; no difference was found in immunohistochemical staining after this additional procedure. A second anti-CAK-β rabbit antibody (anti-CAK-β (C-b)) was used to confirm the specificity of the immunostaining with the first antibody (anti-CAK-β (C-a)). The second antibody was raised against a TrpE fusion protein of rat CAK-β residues 779 to 1008 and was affinity purified on a column of GST fusion protein of the rat CAK-β carboxyl-terminal domain (residues 670 to 1009).[2]

Immunoprecipitation and Immunoblotting of CAK-β

A 2.5% rat brain lysate was prepared on ice by the use of a Teflon pestle in a glass homogenizer in a lysis buffer (20 mmol/L Tris/HCl (pH 7.4), 150 mmol/L NaCl, 2.5 mmol/L EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10% glycerol, 1% aprotinin, 20 mg/ml leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 20 mmol/L $Na_4P_2O_4$). The lysates were subjected to centrifugation at 15,000 × *g* for 20 minutes at 4°C to obtain clarified lysates. CAK-β and α-catenin were immunoprecipitated by mixing anti-CAK-β and anti-α-catenin, respectively, bound to protein A-Sepharose with clarified lysates (1 mg of protein) and by incubation overnight at 4°C on a rotating platform. α-Tubulin and β-tubulin were also immunoprecipitated by mixing anti-α-tubulin and anti-β-tubulin antibodies, respectively, bound to anti-mouse IgG-agarose with clarified lysates (1 mg of protein). The anti-CAK-β, anti-α-catenin, anti-α-tubulin, and anti-β-tubulin beads were prepared for each assay by mixing 3 μg of protein of the affinity-purified anti-CAK-β or 2 μl of anti-α-catenin, anti-α-tubulin, or anti-β-tubulin antibody with 10 μl (packed volume) of protein A-Sepharose or anti-mouse IgG-agarose and washing the Sepharose and agarose beads with the lysis buffer. Immunoprecipitates were washed three times with the lysis buffer, and proteins were separated by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli and Favre.[16] The separated proteins were blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked with 3% bovine serum albumin in TBST (25 mmol/L Tris/HCl (pH 7.5), 150 mmol/L NaCl, and 0.05% Tween 20) for 30 minutes at 60°C and then probed either with affinity-purified anti-CAK-β

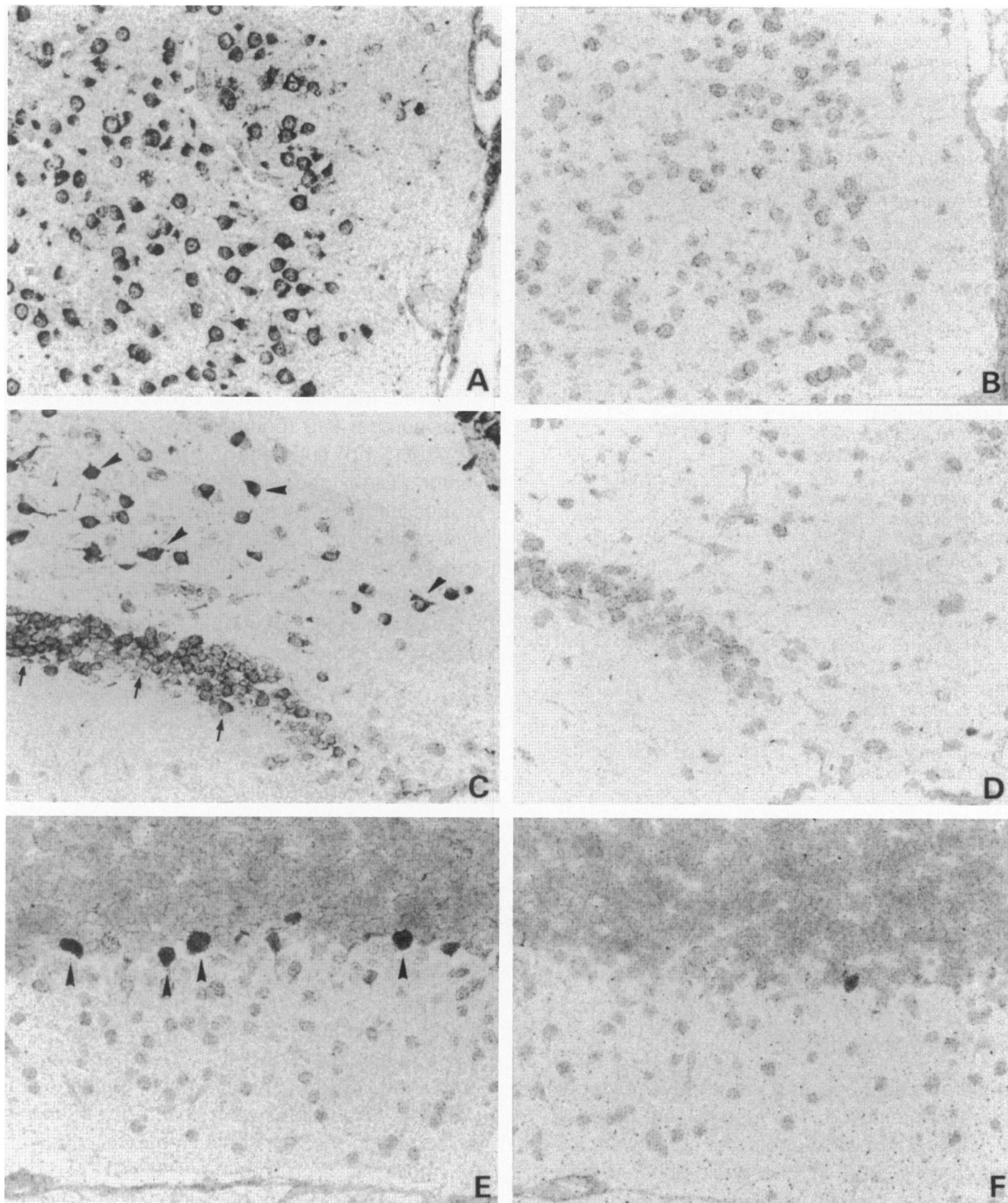

Figure 3. In situ *hybridization in adult rat brain with digoxigenin-labeled RNA probes of the CAK-β mRNA.* **A**: *The cerebral cortex. Darkened cytoplasm indicates the hybridization signals. The large and small pyramidal cells are intensely labeled with the antisense RNA probes.* **B**: *Hybridization with the sense probes in the cerebral cortex in section adjacent to* **A**. *No significant labeling with the sense RNA probes is observed.* **C**: *The hippocampus. The granular neurons* (arrows) *and pyramidal cells* (arrowheads) *are intensely labeled with the antisense RNA probes.* **D**: *Hybridization with the sense probes in the hippocampus in sections adjacent to* **C**. **E**: *Cerebellum. Intense signals of the transcripts are found in the Purkinje cells* (arrowheads). **F**: *Control hybridization in the cerebellum. No particular labeling with sense RNA probes is found. Methyl green was used in counterstaining. Magnification,* ×*165.*

at 1 μg of protein/ml or with anti-α-tubulin or anti-β-tubulin antibody diluted as indicated by the manufacturer in TBST containing 1% bovine serum albumin for 1 hour at room temperature (RT). The membranes were washed with TBST three times and probed again in TBST for 1 hour with monoclonal anti-rabbit immunoglobulins conjugated with alkaline phosphatase, followed by washing

Table 1. *Summary of the Cellular Distribution of Protein and mRNA of CAK-β in Rat Brain and Spinal Cord*

Cell types	Protein	mRNA
Neurons		
Cerebral cortex		
Large pyramidal cells	±	+
Small pyramidal cells	±	+
Granular cells	−	±
Olfactory bulb		
Mitral cells	±	+
Tufted cells	−	−
Hippocampus		
Pyramidal cells	+	++
Gyrus dentatus		
Granular cells	+	+
Pyramidal cells	+	++
Corpus striatum		
Small striatum cells	+	+
Large striatum cells	+	++
Brain stem		
Large cells	+	++
Small cells	+	+
Cerebellum		
Purkinje cells	+	++
Small granular cells	−	−
Large cells in nuclei	+	++
Spinal cord		
Anterior root cells	+	++
Fasciculus cells	+	++
Other types of cells		
Oligodendroglia	±	±
Astrocytes	−	−
Ependymal cells	±	++
Endothelial cells	+	+

three times in TBST. Positive bands were detected by incubation in nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Immunohistochemistry

Rats were deeply anesthetized with Nembutal and organs were removed. The tissues were sliced approximately 5 mm thick, and some slices were fixed with fresh 4% PFA in phosphate-buffered saline (PBS, pH 7.4). The tissues were embedded in paraffin and 5-μm thick sections were mounted on APS-coated glass slides. Other slices mounted in OCT compound were frozen in liquid nitrogen. For immunohistochemistry, the slides were deparaffinized, and the endogenous peroxidase activity was blocked by 0.6% H_2O_2 in methanol for 30 minutes at RT. Block Ace was then used for 30 minutes at RT to avoid nonspecific staining. After being rinsed several times with PBS, primary antibodies were applied on the sections. Anti-CAK-β (C-a) antibody (10 μg protein/ml) was applied overnight at 4°C. Thereafter, we used the avidin-biotin complex method and 3,3′-diaminobenzidine (DAB) as the substrate for peroxidase staining. Either hematoxylin or methyl green was used for counterstaining. As controls, normal rabbit serum was used or primary antibody was omitted from the procedure.

Preparation of RNA Probes for in Situ *Hybridization*

The probe used in the *in situ* hybridization was prepared from rat CAK-β cDNA encompassing base number 813 (counted from the 5′ end) and 1227 (415 bp) subcloned in pBluescript II SK(+) vector. Digoxigenin-labeled single-strand RNA probes in the antisense and sense orientation were synthesized from linearized templates using the appropriate RNA polymerase (T3 or T7 RNA polymerase) in the presence of digoxigenin-labeled UTP (Boehringer Mannheim) according to the manufacturer's protocol.

In Situ *Hybridization*

The materials fixed with 4% PFA were embedded in paraffin. The serial 5-μm-thick sections were mounted on APS-coated glass slides. They were deparaffinized by xylene, rinsed, rehydrated with graded series of ethanol, and dried by cold air. They were then subjected to protease treatment in a solution containing 0.1% pepsin in 0.1 N HCl at RT for 10 minutes. They were refixed with 4% PFA for 10 minutes. After acetylation with 0.25% acetic anhydride (Sigma) in 0.1 mol/L triethanolamine (Sigma), tissue slides were covered with an *in situ* hybridization mixture containing 50% formamide, 0.6 mol/L NaCl, 10 mmol/L Tris/HCl (pH 7.6), 1 mmol/L EDTA, 0.2 mg/ml tRNA, 0.25% SDS, 1× Denhardt's solution, 10% dextran sulfate, and digoxigenin-labeled RNA probes. The digoxigenin-labeled RNA probes in the hybridization mixture were hybridized at 50°C for 36 hours. After hybridization, the tissues were washed in 4× SSC (0.15 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.6) at 50°C and incubated in TNE buffer (10 mmol/L Tris/HCl (pH 7.6), 0.5 mmol/L NaCl, 1 mmol/L EDTA) at 37°C for 10 minutes. After incubation in TNE buffer for 10 minutes, the slides were washed at 50°C in 2× SSC for 30 minutes, in 1×

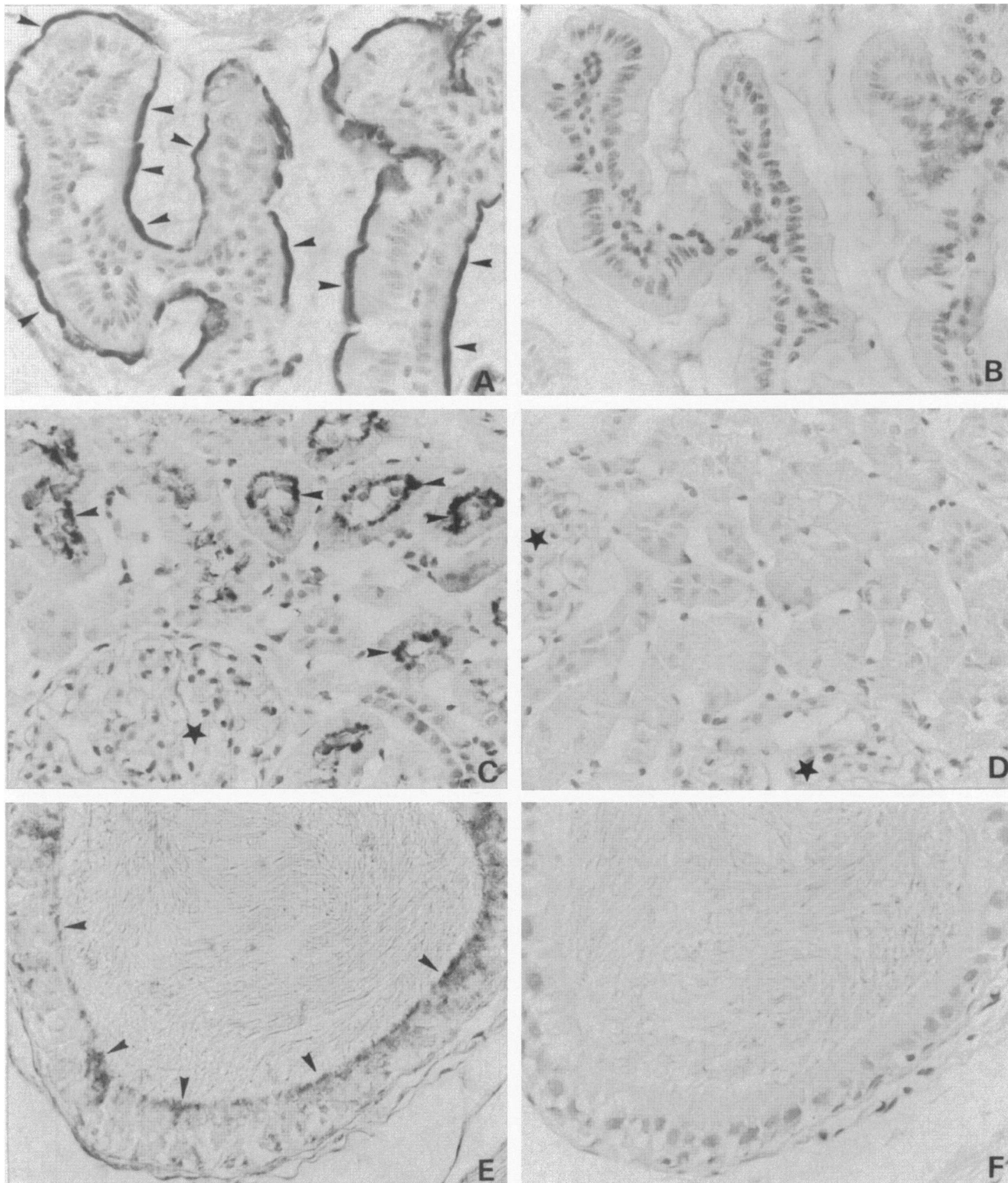

Figure 4. *Expression of CAK-β in various rat tissues.* **A:** *Immunohistochemical staining of small intestine with anti-CAK-β (C-a) antibody. Brush borders of villi are intensely stained* (**arrowheads**). **B:** *Normal rabbit serum is used as a primary antibody. This section is adjacent to that of* **A**. **C:** *Immunohistochemical staining of kidney with anti-CAK-β antibody. Apical membrane of proximal tubular cells are positively immunostained* (**arrowheads**). **Stars** (★) *show the glomerulus.* **D:** *Normal rabbit serum is used as a primary antibody.* **Stars** (★) *show the glomerulus. This section is adjacent to that of* **C**. **E:** *Immunohistochemical staining of rat epididymis with anti-CAK-β antibody.* **Arrowheads** *show the positive staining in cilia of epithelium.* **F:** *Normal rabbit serum is used as a primary antibody. This section is adjacent to that of* **E**. *Hematoxylin was used in counterstaining. Magnification, ×330.*

SSC for 30 minutes, in 0.5× SSC for 30 minutes, and in 0.25× SSC for 30 minutes. Thereafter, tissue slides were incubated with 1% blocking reagent in TN buffer (100 mmol/L Tris/HCl (pH 7.5), 150 mmol/L NaCl) at RT for 30 minutes. After washing twice with TN buffer, anti-digoxigenin polyclonal antibody conjugated with immunogold (30-fold diluted with TN buffer) was applied for 30 minutes and made visible by silver enhancement, followed by counterstaining with methyl green.

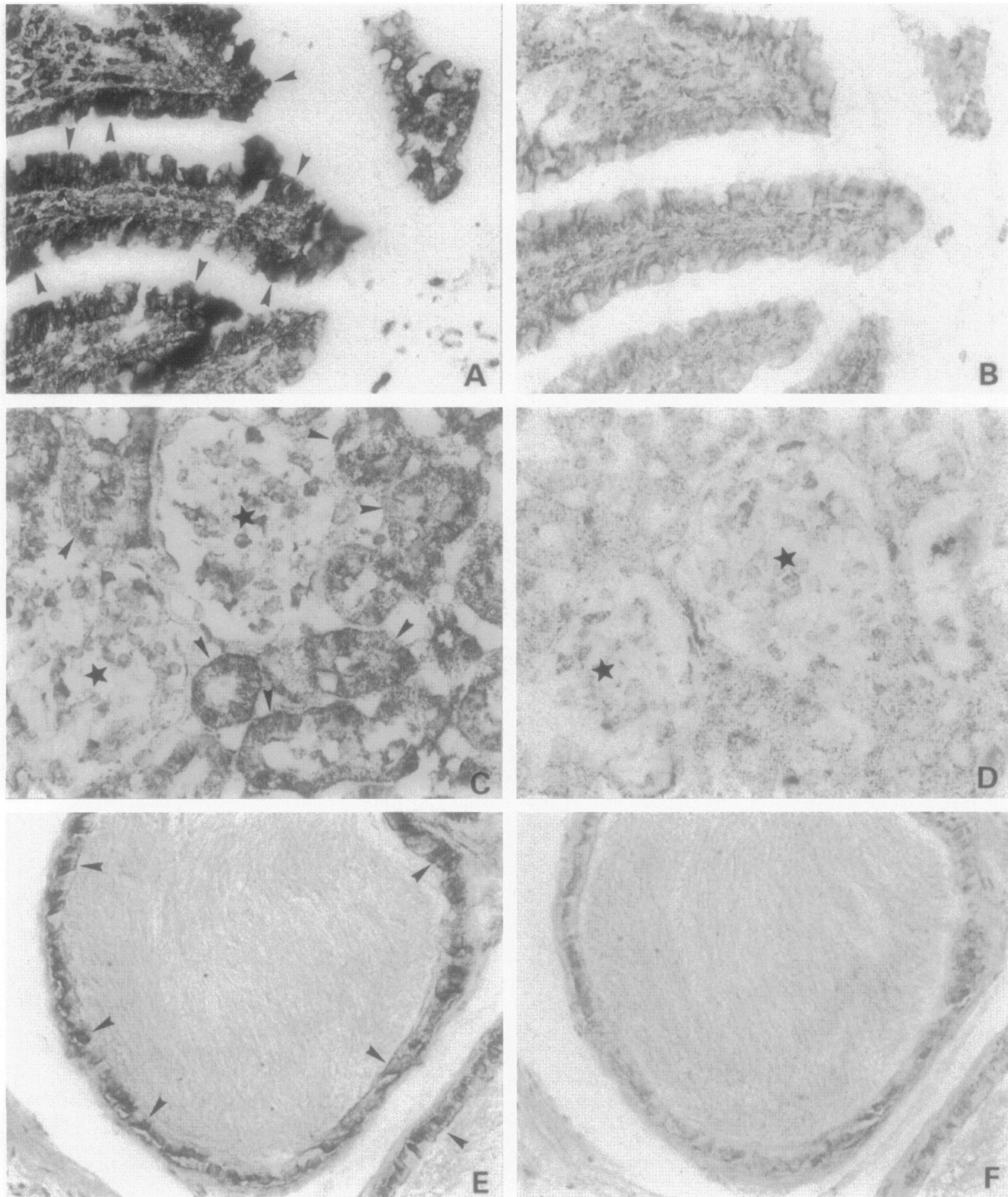

Figure 5. In situ *hybridization of adult rat tissues with digoxigenin-labeled RNA probes of the CAK-β mRNA.* **A:** In situ *hybridization of rat small intestine with digoxigenin-labeled antisense RNA probes of CAK-β. Absorptive epithelial cells are strongly positive for the CAK-β mRNA. Magnification, ×330.* **B:** *Hybridization with the sense probes in the small intestine. This section is adjacent to that of* **A**. *No significant labeling with the sense RNA probes is observed. Magnification ×330.* **C:** In situ *hybridization of rat kidney with an antisense RNA probe of CAK-β. Urinary tubules are labeled with the antisense RNA probe. Magnification, ×165.* **D:** *Hybridization with the sense probes in the kidney. This section is adjacent to that of* **C**. *No significant labeling with the sense RNA probes is observed. Magnification, ×165.* **E:** In situ *hybridization of rat epididymis with an antisense RNA probe of CAK-β. Darkened cytoplasm of ciliated epithelium indicates the presence of CAK-β mRNA. Magnification, ×165.* **F:** *Hybridization with the sense probes in the epididymis. This section is adjacent to that of* **E**. *No significant labeling with the sense RNA probes is observed. Magnification, ×165.*

Table 2. *Summary of the Cellular Distributions of Protein and mRNA of CAK-β in Various Tissues*

Organ and cell types	Protein	mRNA
Heart		
Cardiac muscle	−	ND
Bronchus		
Ciliated cells	+	+
Lung		
Terminal bronchiole	+	+
Alveolar cells	−	±
Esophagus		
Striated epithelium	±	±
Stomach		
Surface epithelial cells	±	±
Fundic glands	±	+
Antral gastric glands	±	+
Small intestine		
Villus epithelium	++	++
Goblet cells	−	−
Paneth cells	−	−
Large intestine		
Absorptive epithelium	+	+
Goblet cells	−	−
Pancreas		
Intercalated duct	−	−
Interlobular duct	−	−
Endocrine cells	−	−
Liver		
Hepatocytes	−	ND
Sinusoidal cells	±	ND
Bile ducts	−	ND
Spleen		
T cells	−	−
B cells	−	−
Kidney		
Glomerulus	−	−
Proximal tubule	++	++
Distal convoluted tubule	±	+
Collecting duct	±	±
Transitional cells	±	±
Salivary gland		
Glandular acinus	−	ND
Striated portion	+	ND
Thyroid		
Follicular cells	+	+
Parathyroid	+	+
Adrenal gland		
Cortex	−	ND
Medulla	−	ND
Testis		
Spermatogonia	−	+
Spermatocytes	−	±
Spermatids	+	±
Spermatozoa	−	−
Sertoli cells	+	+
Leydig cells	+	+
Epididymis		
Efferent ductules	+	++
Ductus epididymis	+	++
Uterus		
Simple columnar epithelium	+	ND
Uterine glands	+	ND
Ovary		
Follicular epithelial cells	−	ND
Lutein cells	±	ND
Oocyte	±	ND
Oviduct		
Ciliated cells	+	ND
Mucous epithelium	±	ND
Placenta		
Syncytiotrophoblast	++	ND
Langerhans' cells	−	ND
Other types of cells		
Endothelial cells	++	++
Striated muscle	−	−
Smooth muscle	−	−
Fat cells	−	−
Cartilage	+	+
Mesothelium	+	+
Connective tissue	+	++

ND, not determined.

Fluorescent Immunostaining and Immunoelectron Microscopy of CAK-β in the Brush Border of Small Intestine

Small pieces of rat small intestine were fixed with 4% PFA in PBS (pH 7.4) for 2 hours and immersed at 4°C in PBS containing 10% sucrose for 4 hours, in PBS containing 15% sucrose overnight, in PBS containing 20% sucrose for 4 hours, and finally in PBS containing 20% sucrose and 5% glycerol for 1 hour. Thereafter, the tissues embedded in OCT compound were frozen in liquid nitrogen and kept at −80°C until use. The thin sections of the frozen samples were mounted on glass slides. The sections were incubated for 30 minutes in cold absolute methanol containing 0.6% H_2O_2. After being rinsed with PBS, the sections were treated with Block Ace for 1 hour. Then, anti-CAK-β antibody was applied to the sections at 20 μg protein/ml overnight at 4°C. After being rinsed with PBS, the sections were incubated overnight at 4°C either with anti-rabbit Ig conjugated with fluorescein isothiocyanate or with anti-rabbit IgG conjugated with peroxidase. Fluorescent-stained samples were incubated with rhodamine-conjugated phalloidin for 30 minutes at RT. After being rinsed with PBS, the sections were mounted with 90% glycerol containing 1 mg/ml *p*-phenylene diamine. Digital images of the fluorescence distribution in the cells of small intestine were obtained using a Zeiss Axioskop 20 microscope equipped for epifluorescence (100× objective lens). Images at various planes were obtained with a computer-controlled focus mechanism and a Photometrics CH250 thermoelectrically cooled CCD (Scanalytics CELLscan system, Scanalytics, Billerica, MA).

The samples incubated with peroxidase-conjugated antibody were rinsed with PBS and refixed with 1.25% glutaraldehyde for 20 minutes. After be-

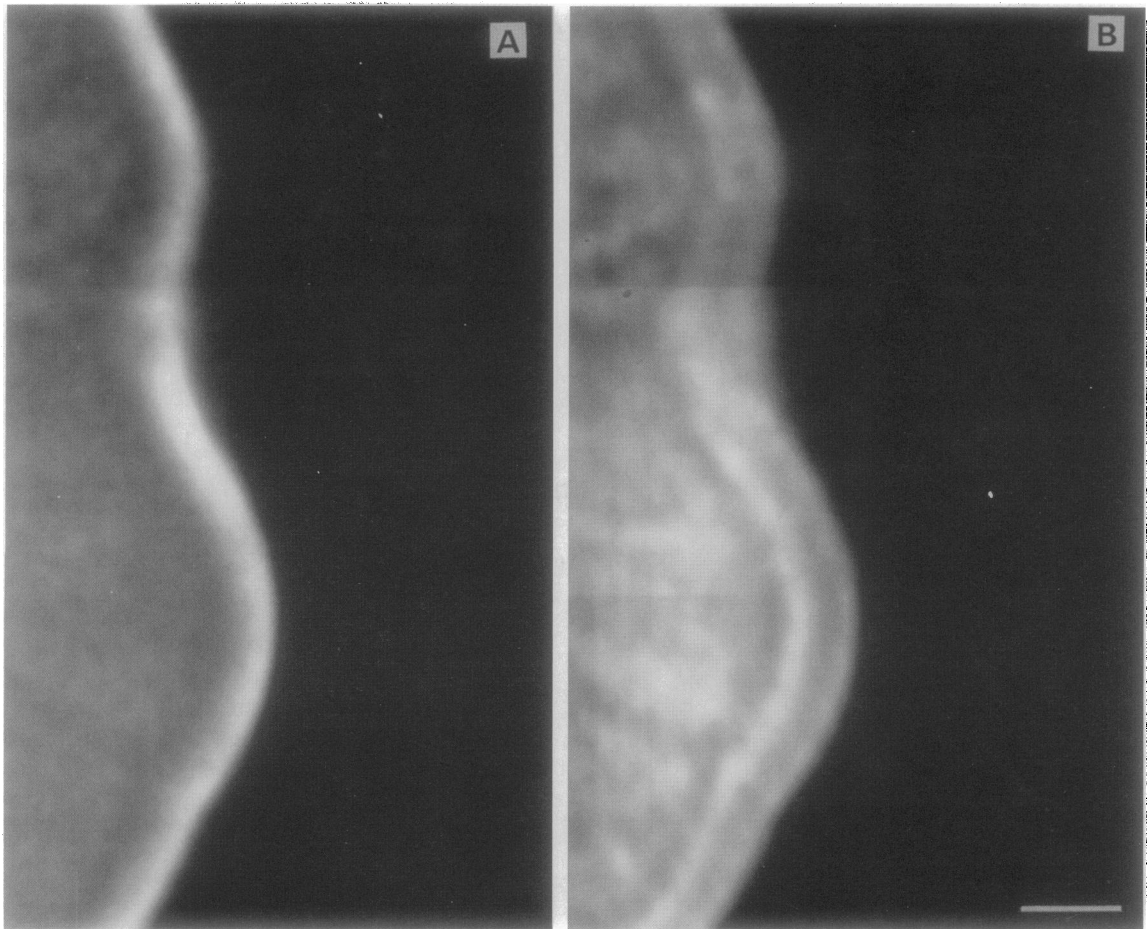

Figure 6. *Double labeling of CAK-β (A) and actin (B) in rat small intestine. Actin was visualized using rhodamine-conjugated phalloidin, and CAK-β was visualized using a rabbit anti-CAK-β (C-a) antibody and fluorescein-isothiocyanate-conjugated anti-rabbit Ig antibody. Brush borders of intestinal epithelial cells are intensely stained. Bar, 2 μm.*

ing rinsed with PBS, the sections were incubated for 5 minutes with 1 mg/ml DAB in 0.05 mmol/L Tris/HCl (pH 7.6) containing 0.03% H_2O_2. Thereafter, the samples were postfixed in 2% osmium tetroxide in the buffer, dehydrated, and embedded *in situ* in Epon 812 for electron microscopy.

Results

Specificity of the Anti-CAK-β Antibodies

The same immunohistochemical staining was obtained by the use of the two different anti-CAK-β rabbit antibodies. One rabbit antibody (anti-CAK-β (C-a)) was directed to 47 amino acid residues from the very end of the catalytic domain to the first 38 residues of the carboxyl-terminal domain, residues 670 to 716 of CAK-β. All results presented in this paper except Figure 1 were obtained with this antibody. The other rabbit antibody (anti-CAK-β (C-b)) was directed against the carboxyl-terminal 230 residues of CAK-β, residues 779 to 1008. These two antibodies specifically immunoprecipitated CAK-β from the rat brain (Figure 1A). The specificity of these antibodies to CAK-β was also examined by probing immunoblots of total brain proteins (Figure 1B). In the immunoblot, CAK-β and a band below CAK-β were found. Preliminary results indicate that the lower band is a degraded CAK-β. The same immunohistochemical results obtained with these two antibodies directed to different portions of CAK-β indicated that the immunostaining we observed was specific to CAK-β.

CAK-β in Adult Rat Brain

We first examined the expression of CAK-β protein by immunohistochemistry in an adult rat brain, as the rat CAK-β cDNA clone was isolated from an adult rat brain cDNA library and the CAK-β gene transcripts

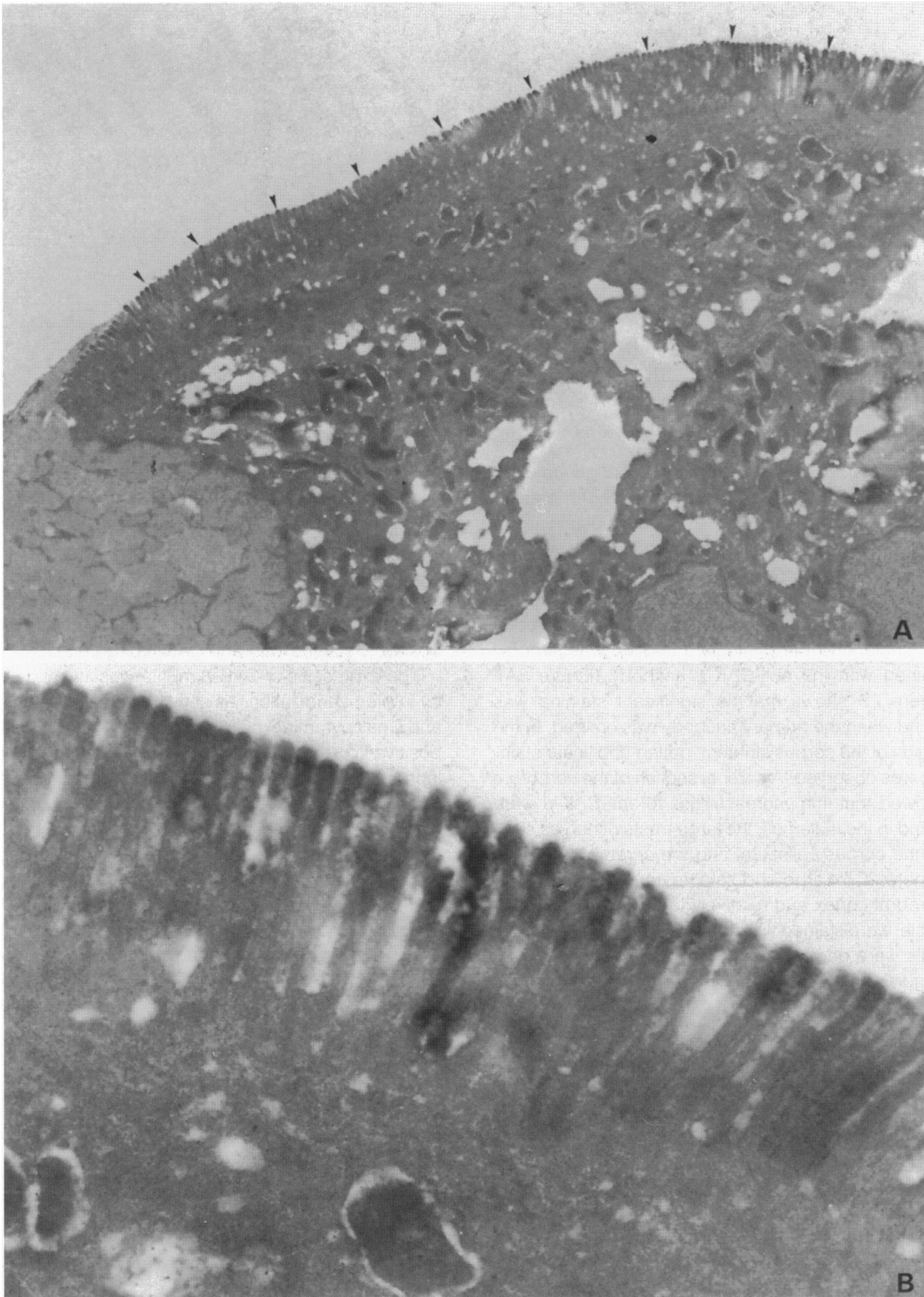

Figure 7. *Immunoelectron micrographs of CAK-β in rat small intestine.* A: *High-density structures* (arrowheads) *are observed in the apical membrane of intestinal absorptive cells. Goblet cells are not positively immunostained. Magnification, ×8000.* B: *Enlargement of the apical portion shown in* A. *DAB-positive granules are present within the microvilli of intestinal brush borders. Magnification, ×40,000.*

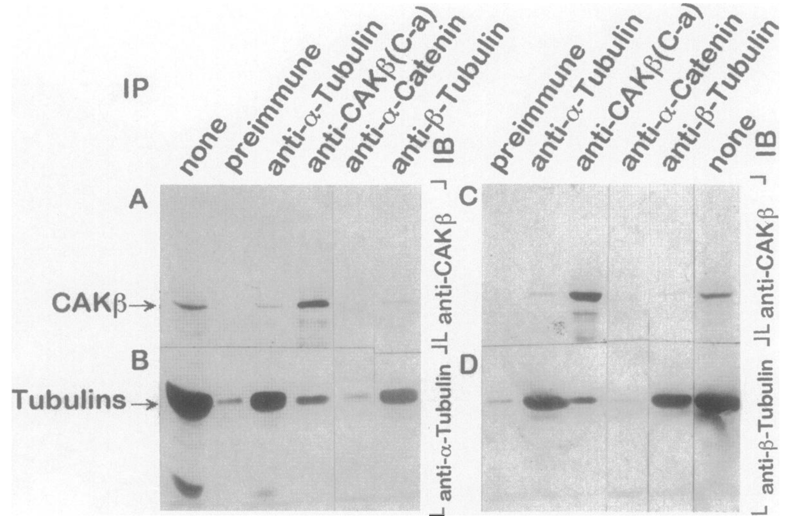

Figure 8. *Co-immunoprecipitation of CAK-β and tubulins from rat brain lysate. Immunoprecipitation (IP) was done with the indicated antibody bound to Sepharose or agarose beads. To the lanes marked* none*, 1 mg of protein of total rat brain lysate was applied in the SDS-polyacrylamide gel electrophoresis. After blotting onto polyvinylidene difluoride membranes, the membranes were cut into upper and lower parts. The upper part of the membrane was probed with anti-CAK-β. The lower part of the membrane was probed either with anti-α-tubulin or with anti-β-tubulin as indicated. IB, immunoblotting.*

are most abundant in the brain.[2] Although we used 4% PFA in PBS (pH 7.4) as a fixative in the present study, we confirmed positive immunostaining of CAK-β in the materials fixed with neutral buffered 10% formalin and with absolute ethanol. A sagittal section of the rat brain revealed that the medulla, especially the corpus callosum, optic tract, cerebral peduncle, and the geniculate bodies, was strongly stained with the anti-CAK-β antibody (Figure 2A). Figure 2B shows that no significant staining was found when the primary antibody was omitted. In the region of the corpus striatum, dotted and linear staining was observed. A coronal section of the cerebrum showed that immunoreactivities to anti-CAK-β were found in the thalamus, the large tracts of nerves such as the corpus callosum, and the cerebral peduncle (Figure 2C). Large and small pyramidal cells in the cerebral cortex and relatively large neurons in most nuclei were stained with anti-CAK-β (Figure 2E). Glial cells were negative in the staining with anti-CAK-β (Figure 2E). Endothelial cells and ependymal cells were positively stained. In the cerebellum, the medulla was also strongly stained with anti-CAK-β (Figure 2, A and F). Purkinje cells were positively stained with anti-CAK-β, whereas neurons in the granular layers were not stained (Figure 2F). Large neurons in the nuclei of the cerebellum and the brain stem were also positively stained. In addition, both anterior root neurons and white matter in the spinal cord were positively stained with anti-CAK-β (Figure 2D).

The expression of CAK-β mRNA was investigated by *in situ* hybridization. As shown in Figure 3A, large and small pyramidal cells in the cerebral cortex were positive for the hybridization signals, which were located in the perikaryon. The corresponding sense probe hybridized on the adjacent sections did not give any distinct hybridization signal (Figure 3, B, D, and F), demonstrating the specificity of the antisense probe used in this *in situ* hybridization. In the hippocampus, pyramidal and granular cells strongly expressed mRNA in their cytoplasm (Figure 3C). Glial cells except for ependymal cells were not clearly positive for the expression of CAK-β mRNA. Although Purkinje cells in the cerebellum were la-

Table 3. *Relationship between CAK-β Expression and Associated Cellular Structures*

Organs	Types of cells	Cellular structures	Associated structures (suspected)
Bronchus	Ciliated cells		
Epididymis	Ciliated duct cells	Cilia	Microtubules
Oviduct	Ciliated cells		
Testis	Spermatids	Flagella	Microtubules
Nervous system	Neurons	Axon	Microtubules
Intestine	Villous epithelium		
Kidney	Proximal tubules	Brush border	Microfilaments
Vessels	Endothelial cells		
Placenta	Syncytiotrophoblasts	Apical microvilli	Microfilaments
Choroidal plexus	Ependymal cells		
Serous membrane	Mesothelial cells		

beled with the probe, granular cells did not show any distinct hybridization signal (Figure 3E). In the spinal cord, most neurons in the gray matter strongly expressed CAK-β mRNA (data not shown). The cellular distributions of CAK-β protein and mRNA in the central nervous system are summarized in Table 1. Although most neurons expressed CAK-β mRNA, the immunoreactivity to anti-CAK-β antibody was relatively weak in the perikarya of the cells but was strong in their axons.

Expression of CAK-β in Other Tissues

Various tissues of an adult rat were immunostained for CAK-β. Figure 4 shows representative tissues. In the small intestine, CAK-β localized in the brush border in villi (Figure 4A). Goblet cells were negative in the immunostaining. In the kidney, the immunoreaction to anti-CAK-β was found in the proximal tubules, especially in the apical membrane of the tubular cells (Figure 4C). Glomerulus and distal tubules did not show any positive staining. In the epididymis, CAK-β protein was concentrated in the cilia, which were present in the apical membrane of the ductular cells (Figure 4E). Normal rabbit serum (Figure 4, B, D, and F) did not show any distinct staining in each adjacent section of Figure 4, A, C, and E, respectively.

In situ hybridization of antisense- and sense-oriented RNA probes of CAK-β was carried out to examine the expression of the mRNA in the representatives of various rat tissues. In the small intestine, the CAK-β mRNA was strongly expressed in the cytoplasm of absorptive epithelial cells (Figure 5A). In the kidney, the CAK-β mRNA expression was much more prominent in the cells of the proximal tubules than in those of the distal tubules (Figure 5C). In the epididymis, hybridization signals with antisense CAK-β RNA probes were observed in the cytoplasm of the ciliated cells (Figure 5E). The corresponding sense probe hybridized on the adjacent sections did not give any distinct hybridization signal (Figure 5, B, D, and F), demonstrating the specificity of the antisense probe used in this *in situ* hybridization. The cellular distribution of the protein and mRNA of CAK-β in various tissues is summarized in Table 2.

Double Staining for CAK-β and Actin of Small Intestine

It is well known that the brush border of small intestinal epithelium contains actin filaments. Therefore, to prove that CAK-β in absorptive epithelial cells localize at the brush border, we carried out the double staining for CAK-β and actin filament. Figure 6A shows an intense belt-like staining of CAK-β in the apical membrane of the cells. A positive staining for rhodamine-phalloidin was observed in the same location (Figure 6B), which indicates the concentration of CAK-β at the brush border of the cells.

Immunoelectron Microscopy for CAK-β of Small Intestine

Immunoelectron microscopy of the rat small intestine revealed that a high-density substance representing the immunoreactivity to CAK-β was apparently located in the apical surface of absorptive cells (Figure 7A). An enlargement of the darkened regions revealed that the DAB-positive substance was localized within the microvilli of absorptive cells (Figure 7B).

Co-Immunoprecipitation of CAK-β and Tubulins from Rat Brain Lysate

Immunostaining in various tissues suggested an association of CAK-β with cytoskeletal proteins such as actin, actin-associated proteins, and tubulins. The association of CAK-β with tubulins was demonstrated by co-immunoprecipitation of CAK-β and tubulins from rat brain lysate (Figure 8). Rat brain lysate was subjected to immunoprecipitation with anti-CAK-β (C-a), anti-α-tubulin, anti-β-tubulin, and anti-α-catenin antibodies (Figure 8). The immunoprecipitates were analyzed by immunoblotting with anti-CAK-β (C-a), anti-α-tubulin, and anti-β-tubulin. In the immunoprecipitates with anti-CAK-β, a significant enrichment of α- and β-tubulins was observed when compared with the tubulins found in the immunoprecipitates with preimmune serum and with anti-α-catenin rabbit serum (Figure 8). A small amount of CAK-β was also found in the immunoprecipitates with anti-α-tubulin and with anti-β-tubulin. Although our attempts to prove co-immunoprecipitation of CAK-β and actin from tissue lysates did not give us a positive result, we were able to show co-immunoprecipitation of CAK-β and actin with anti-CAK-β from lysates of cultured cells (data not shown).

Discussion

In this paper, we examined the CAK-β expression in various tissues. For this purpose, the results of immunohistochemistry for CAK-β were combined

with those of *in situ* hybridization for CAK-β mRNA expression. The immunohistochemical study revealed that CAK-β was concentrated in certain cell structures. The relationship between the intracellular localization of CAK-β and the corresponding cell structures present in this location is summarized in Table 3. The cells with cilia and axons were highly positive for CAK-β mRNA. CAK-β protein was present at high levels in these cell structures. These results suggest an association of CAK-β with microtubules. Co-immunoprecipitation of CAK-β and tubulins with anti-CAK-β antibodies provides evidence confirming the association of CAK-β with microtubules. CAK-β was also found to be concentrated in microvilli in small intestinal epithelial cells and renal proximal tubular cells; these cells with microvilli expressed high levels of the CAK-β mRNA. Although we were not able to find actin or actin-associated proteins in the immunoprecipitates from rat brain lysate with anti-CAK-β antibody, fluorescent immunohistochemistry and immunoelectron micrography indicate an association of CAK-β with microfilaments. In fact, we have co-immunoprecipitated actin and CAK-β with anti-CAK-β from lysates of cultured cells (manuscript in preparation). Thus, CAK-β was localized in specific cell structures containing microtubules and microfilaments, and CAK-β might play important roles in their functions, although most cells may express CAK-β to some extent.

FAK was originally identified in Src-transformed fibroblasts, where it is predominantly expressed at focal adhesions.[17,18] Recently, Grant et al[19] reported that FAK was expressed at very high levels in the brain and was not restricted to focal adhesions in the neural tissues. FAK was found in axons and dendrites in neurons and also in the intermediate filament cytoskeleton of astrocytes. Therefore, the association with intracellular structures is probably not unique to CAK-β but common to CAK-β and FAK. We have studied the subcellular distribution of CAK-β in various cultured cell lines (manuscript in preparation). CAK-β was present at the site of cell-to-cell contacts in some cell lines such as MDCK and Caco-2, as we showed previously in the CAK-β cDNA-transfected COS7 cells,[2] but this subcellular localization of CAK-β was not always the case. The results on cell lines have been in accord with the results shown in this paper. It has been proposed that the activation of Ras and mitogen-activated protein kinase (MAPK) by a Ca^{2+}-dependent pathway is one important function of CAK-β.[4,20] Mitogen-activated protein kinase is present in dendrites and colocalizes with microtubules. The association of CAK-β with microtubules may be possibly important in activating the Ras/mitogen-activated protein kinase pathway.

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