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MOLECULAR CLONING OF RAT RAC PROTEIN KINASE α AND β AND THEIR ASSOCIATION WITH PROTEIN KINASE C ζ *

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SUMMARY: cDNAs of RAC protein kinases (RAC-PK) α and β were cloned from a rat testis cDNA library. The predicted open reading frames encode 480 and 481 amino acids of RAC-PK α and β, respectively, and the rat RAC-PK α and β have sequences conserved among different mammalian species such as the pleckstrin homology domain at their amino-terminal region and the protein-serine/threonine kinase catalytic domain at their carboxyl-terminal region. RNA blot analysis showed wide distribution of two RAC-PK in rat tissues. Immunoprecipitation analysis revealed that RAC-PK α and β associate with protein kinase C ζ through the pleckstrin homology domain *in vitro*, suggesting the interaction between RAC-PK and protein kinase C.

Protein phosphorylation is regarded to play a central role in intracellular signal transduction through regulation of the function of a variety of proteins. cDNAs for more than one hundred of protein kinases have been isolated from various eukaryotic species (1). The protein kinases share a related catalytic domain, and the members of the enzyme family are

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Abbreviations: PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PCR, polymerase chain reaction; RAC-PK, related A and C kinase; PH domain, pleckstrin homology domain; SH2 domain, *src*-homology 2 domain.

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classified into subfamilies based on amino acid sequence comparisons within the catalytic domain (2). Many protein kinases have been shown to exist as groups of subtypes rather than a single enzyme, such as cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). A protein-serine/threonine kinase, which has a catalytic domain closely related to both PKA and PKC, was identified independently by three groups, and therefore designated as rac (3), pkb (4,5), and *Akt* (6), respectively. Namely, Hemmings' group first isolated the cDNA clone termed rac (related to A and C kinases) by screening of cDNA libraries derived from the human cell lines MCF-7 and WI38 under low-stringent conditions using a DNA fragment encoding the catalytic subunit of PKA as a probe (3). Coffer and Woodgett (4,5) employed PCR using highly degenerated oligonucleotide primers designated from regions conserved among protein-serine/threonine kinases to amplify the human epithelial HeLa cell cDNA library, and finally obtained the clone named pkb by screening human fibroblast and bovine brain libraries with the PCR fragment. On the other hand, *Akt* was identified as a rodent retroviral oncogene (6), and later c-*akt* was isolated from murine thymus cDNA library by using v-*akt* as a probe by Tsichilis and colleagues (7). These homologous clones isolated from different mammalian species encode a protein-serine/threonine kinase domain closely related to those of the PKC and PKA families as described above. Later, a second form of this protein kinase was identified from human fibroblast (8) and thymus (9) libraries. Thus, these clones appear to represent a new group of a protein-serine/threonine kinase. In this report, we call these two clones as RAC-PKα and RAC-PK β, respectively, according to Hemmings' group (3,8), as they first reported these clones.

Recently, a new region of homology has been identified in many proteins known to play roles in signal transduction such as protein kinases, phospholipases, and GTP-binding proteins (10-14). This domain has been designated as the pleckstrin homology domain (PH domain), as it has originally found in pleckstrin, the major PKC substrate of platelets (10). The PH domain is suggested to participate in protein-protein interactions involved in signal transduction. The amino-terminal region of RAC-PK contains the PH domain that may function in a regulatory role of this protein kinase family [1]. It has not been clear, however, whether the amino-terminal region of the RAC-PK containing the PH domain binds to a specific protein. We report here the molecular cloning of RAC-PK α and β from a rat cDNA library, and association of RAC-PK α and β with PKC ζ *in vitro*.

EXPERIMENTAL PROCEDURES

Isolation of cDNA Clones: Poly(A)$^+$RNA prepared from eight-week old SD rats was primed with an oligo(dT) primer, and the first strand cDNA was synthesized with avian myeloblastosis virus reverse transcriptase (BRL, Gaithersburg, MD) as a template cDNA for PCR. Fully degenerated oligonucleotides deduced from the amino acid sequences of the rat PKC, that are highly conserved among the family (16), were synthesized as primers for PCR.

[1] The amino-terminus of RAC-PK was once reported to contain a region similar to the *src* homology-2 (SH2) domain (6,7), that is a structural motif functions in protein-protein interactions recognizing phosphotyrosine (15), but later the region was regarded to be an example of the PH domain (10).

5' Sense primer = 5'-GTTTAAGCTTAAA/GGGNAGT/CTTT/CGGNAA-3'
3' Antisense primer = 5'-GAAAGGATCCTTA/GTCNAA/GT/CTTNAA/GA/GTC-3'
The sense primer corresponds to the amino acid residues 347 to 352 of the α-subspecies of the rat PKC (Lys-Gly-Ser-Phe-Gly-Lys) with a *Bam*HI restriction site and four additional bases (GTTT) on its 5' end, whereas the antisense primer corresponds to the amino acid residues 463 to 468 of the subspecies (Asp-Leu-Lys-Leu-Asp-Asn) with a *Hind*III restriction site and four additional bases (GAAA) on its 5' end. The reaction was carried out under the conditions described (17), with 25 cycles of denaturation (1 min at 94°C), annealing (2 min at 50°C), and polymerization (3 min at 72°C). The amplified products were digested with *Bam*HI and *Hind*III, subcloned into pUC19 vector, and sequenced by dideoxy chain-termination method using DNA Sequencing System Model 373A (Applied Biosystems, Foster City, CA). The cloned fragment containing RAC-PK α was excised from the plasmid, labeled with [α-^{32}P]dCTP, and employed as a probe for the plaque hybridization screening of a rat testis cDNA library in λZAP II (Stratagene, La Jolla, CA). Hybridization proceeded under high stringency in 6 X SSC containing 5 X Denhardt's solution, 100 μg/ml heat-denatured non-homologous DNA, and 1% SDS at 65°C for 16 h. Post-hybridization proceeded in 0.2 X SSC containing 0.1% SDS at 65°C for 30 min. Nine positive phages were isolated from about 4 X 10^5 recombinants, and the cloned cDNAs were excised *in vivo* as a pBluescript form by using a helper phage R408. Three clones out of the nine positive clones included RAC-PK α, and other six clones encoded RAC-PKβ. The clones with the longest insert for RAC-PK α (pRAC101) and RAC-PK β (pRAC102) were employed in the following studies.

RNA Blot Analysis: The RNA blot containing mRNAs from several rat tissues was obtained from Clontech Laboratories (Palo Alto, CA). The probes for detection of specific RAC-PK subspecies were prepared from 3' none-coding region of cDNAs, *Sac*I-*Eco*RI fragment of the RAC-PK α and the *Eco*RI-*Bam*HI fragment of the RAC-PK β were labeled with [α-^{32}P]dCTP and used as probes. Hybridization and post-hybridization were carried out under the conditions described by the manufacturer, and the radioactivity in hybridized bands was analyzed by Bio-imaging Analyzer BAS2000 (Fuji, Tokyo, Japan).

Preparation of Antisera: Antisera against the RAC-PK were raised using the rat RAC-PK α protein expressed in *E. coli* as an antigen. The RAC-PK α protein was mixed with Freund's complete adjuvant and injected subcutaneously into rabbits. Freund's incomplete adjuvant was used for secondary immunization and the later boosts. The rabbit antiserum CKpV5ζ-a that specifically recognizes the carboxyl-terminus of PKC ζ was raised as described (18).

Expression of RAC-PK and PKC ζ: The cDNA inserts of RAC-PK α (pRAC101) and RAC-PK β (pRAC102) were separately introduced into the *Eco*RI site of the expression vector pTB701 (19), and the plasmids constructed were referred to as pRAC201 and pRAC202 for each RAC-PK α and RAC-PK β, respectively. COS-7 cells were transfected with the plasmid DNA by electroporation using GENE PULSAR (Bio Rad). CHO cells overexpressing PKC ζ were cultured as previously described (20).

Immunoprecipitation and Immunoblot Analysis: Cells were washed with phosphate-buffered saline, and lysed in 20 mM Tris-HCl at pH 7.5 containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1% Triton X-100, 150 mM NaCl, 50 mM NaF and 1 mM phenylmethylsulfonyl fluoride. After centrifugation for 30 min at 100,000 X g, the supernatant was recovered as an extract. Cell extracts from COS-7 cells and CHO cells were combined and incubated for 1 h at 4°C, and then incubated with 2 μl of the antiserum CKpV5ζ-a for 2h at 4°C. Immune-complexes were collected by using protein A-Sepharose beads (Pharmacia). Immunoprecipitates were washed four times with 10 mM Tris-HCl at pH 7.5 containing 0.5 M NaCl, and 1% Triton X-100, boiled in the sample buffer for SDS-PAGE (21). After SDS-PAGE, proteins were transferred onto an Immobilon P membrane (Millipore), and immunoblot analysis was carried out using the antiserum against RAC-PK as the first antibody and the alkaline phosphatase-conjugated anti-rabbit second antibody (Promega) as described (20).

RESULTS AND DISCUSSION

Several oligonucleotide primers degenerate from regions conserved among the rat PKC subspecies were employed for PCR to isolate novel cDNAs related to the PKC family. DNA fragments of the expected size range were subcloned, that are amplified from the rat testis

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A                                        GAATTCCCTGGGTTACCCCGGTGTGTGGGGGCCACGGATACC    -1
   ATGAACGACGTAGCCATTGTGAAGGAGGGCTGGCTGCACAAACGAGGGGAATATATTAAAACC    63
1    M  N  D  V  A  I  V  K  E  G  W  L  H  K  R  G  E  Y  I  K  T
   TGGCGGCCACGCTACTTCCTCCTCAAGAATGATGGCACCTTTATTGGCTACAAGGAACGGCCT   126
22   W  R  P  R  Y  F  L  L  K  N  D  G  T  F  I  G  Y  K  E  R  P
   CAGGATGTGGAGCAGCGCGAGTCCCCACTCAACAACTTCTCAGTGGCACAATGTCAGCTGATG   189
43   Q  D  V  E  Q  R  E  S  P  L  N  N  F  S  V  A  Q  C  Q  L  M
   AAGACAGAGCGGCCGAGGCCCAACACCTTCATCATCCGCTGCCTGCAGTGGACCACGGTCATT   252
64   K  T  E  R  P  R  P  N  T  F  I  I  R  C  L  Q  W  T  T  V  I
   GAGCGCACCTTCCATGTGGAAACGCCTGAGGAGCGGGAAGAGTGGACCACCGCCATTCAGACT   315
85   E  R  T  F  H  V  E  T  P  E  E  R  E  E  W  T  T  A  I  Q  T
   GTGGCTGATGGACTCAAACGGCAGGAGGAGGAGACGATGGACTTCCGGTCAGGTTCACCCAGT   378
106  V  A  D  G  L  K  R  Q  E  E  E  T  M  D  F  R  S  G  S  P  S
   GACAACTCAGGTGCTGAGGAGATGGAGGTGGCCCTGGCCAAGCCCAAGCACCGTGTGACCATG   441
127  D  N  S  G  A  E  E  M  E  V  A  L  A  K  P  K  H  R  V  T  M
   AACGAGTTTGAGTACCTGAAGCTACTGGGCAAGGGCACCTTTGGGAAGGTGATCCTGGTGAAG   504
148  N  E  F  E  Y  L  K  L  L  G  K  G  T  F  G  K  V  I  L  V  K
   GAGAAGGCCACAGGTCGCTACTATGCCATGAAGATCCTCAAGAAGGAGGTCATCGTTGCCAAG   567
169  E  K  A  T  G  R  Y  Y  A  M  K  I  L  K  K  E  V  I  V  A  K
   GATGAGGTTGCCCACACGCTTACTGAGAACCGTGTCCTGCAGAACTCTAGGCATCCCTTCCTT   630
190  D  E  V  A  H  T  L  T  E  N  R  V  L  Q  N  S  R  H  P  F  L
   ACAGCCCTCAAGTACTCATTCCAGACCCACGACCGCCTCTGCTTTGTCATGGAGTACGCCAAT   693
211  T  A  L  K  Y  S  F  Q  T  H  D  R  L  C  F  V  M  E  Y  A  N
   GGAGGCGAGCTCTTCTTCCACCTGTCTCGTGAGCGCGTGTTTTCAGAGGACCGGGCCCGCTTC   756
232  G  G  E  L  F  F  H  L  S  R  E  R  V  F  S  E  D  R  A  R  F
   TACGGTGCGGAGATTGTGTCCGCCCTGGACTACTTGCACTCCGAGAAGAACGTGGTGTACCGG   819
253  Y  G  A  E  I  V  S  A  L  D  Y  L  H  S  E  K  N  V  V  Y  R
   GACCTCAAGCTGGAGAACCTCATGCTGGACAAGGACGGGCACATCAAGATAACGGACTTCGGG   882
274  D  L  K  L  E  N  L  M  L  D  K  D  G  H  I  K  I  T  D  F  G
   CTGTGCAAGGAGGGTATCAAGGACGGTGCCACCATGAAGACGTTCTGCGGGACACCCGAGTAC   945
295  L  C  K  E  G  I  K  D  G  A  T  M  K  T  F  C  G  T  P  E  Y
   CTGGCCCCTGAGGTGCTGGAGGACAACGACTATGGCCGTGCAGTGGACTGGTGGGGGCTGGGC  1008
316  L  A  P  E  V  L  E  D  N  D  Y  G  R  A  V  D  W  W  G  L  G
   GTGGTCATGTACGAGATGATGTGCGGCCGCCTGCCCTTCTACAACCAGGACCATGAGAAGCTG  1071
337  V  V  M  Y  E  M  M  C  G  R  L  P  F  Y  N  Q  D  H  E  K  L
   TTCGAGCTCATCCTAATGGAGGAGATCCGCTTCCCACGCACACTCGGGCCGGAGGCCAAGTCC  1134
358  F  E  L  I  L  M  E  E  I  R  F  P  R  T  L  G  P  E  A  K  S
   CTGCTCTCGGGGCTGCTCAAGAAGGACCCTACACAGAGGCTCGGTGGGGGCTCCGAGGACGCC  1197
379  L  L  S  G  L  L  K  K  D  P  T  Q  R  L  G  G  G  S  E  D  A
   AAGGAGATCATGCAGCACCGCTTCTTTGCCAACATCGTGTGGCAAGATGTGTATGAGAAGAAG  1260
400  K  E  I  M  Q  H  R  F  F  A  N  I  V  W  Q  D  V  Y  E  K  K
   CTGAGCCCACCTTTCAAGCCCCAGGTCACCTCTGAGACCGACACCAGGTATTTTGATGAGGAG  1323
421  L  S  P  P  F  K  P  Q  V  T  S  E  T  D  T  R  Y  F  D  E  E
   TTCACAGCTCAGATGATCACCATCACACCGCCTGATCAAGATGACAGCATGGAGTGTGTGGAC  1386
442  F  T  A  Q  M  I  T  I  T  P  P  D  Q  D  D  S  M  E  C  V  D
   AGTGAACGGAGGCCGCACTTTCCCCAGTTCTCCTACTCAGCCAGTGGCACAGCCTGAGGCCTG  1449
463  S  E  R  R  P  H  F  P  Q  F  S  Y  S  A  S  G  T  A End
   GGGTGGTGGCTGGCAGCCCTGCACCCCTCTGCATTGCCGAGTCCAGAAGCCCCGCATGGATCA  1512
   TCTGTACCTGATGGTTTTGTTTCTCGGATGTGCTGGGGAGGAACCTCGCCAGCCTCGGAATTC  1575
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Fig. 1. Nucleotide and deduced amino acid sequences. A, RAC-PK α (pRAC101). RAC-PK α consists of 480 amino acids with a calculated molecular weight of 55,731. B, RAC-PK β (pRAC102). RAC-PK β consists of 481 amino acids with a calculated molecular weight of 55,023. Nucleotide residues are numbered beginning with the first ATG triplets encoding the putative initiating methionines, and those positions are indicated at the right. Deduced amino acid residues are given in the single-letter code under the nucleotide sequences, and those positions are indicated at the left. Nucleotides 472 to 837 of the pRAC101 correspond to the PCR-amplified fragment, and nucleotides 472 to 488 and 820 to 837 correspond to the PCR primers, showing a perfect match to the primers except for nucleotide 479 and 834.

cDNA by combination of the primers based on the subdomains I and VIB of the protein kinase catalytic domain (2). The nucleotide sequencing revealed that one of the amplified DNA fragments shows high homology with the human RAC-PK (3), which was available when this

```
B                                                    GCCGCTTAGGAAGCCTTCAGTTTGGATCC -253
     CAAGGCCTCCTTGATCCTGCACCTGGAGCTCTGTTAGCACCGTTCTTTCTGAGTCCTGTTTCT -190
     GCGCGTGCTACGCTTACGCACCTGCCTCCTTGGGCACTCAAGCTCGGAACTGGATTTCCACTG -127
     ACCTTGTATCTGATTTTCTAAAAGGCCTTCACTAAATTAAGACTATACTGTATCGGAGGAGCC  -64
     TCCTACATGTCCTGCTGCCCTGAGCTCTCTCAAGCTAGGTGACAGCGTGTTAATGCTGCCACC   -1
     ATGAATGAGGTATCTGTCATCAAAGAAGGCTGGCTCCACAAACGTGGTGAATACATCAAGACC   63
  1  M  N  E  V  S  V  I  K  E  G  W  L  H  K  R  G  E  Y  I  K  T
     TGGAGGCCCCGGTACTTCCTTCTGAAGAGTGATGGATCTTTTATTGGGTATAAAGAGAGACCT  126
 22  W  R  P  R  Y  F  L  L  K  S  D  G  S  F  I  G  Y  K  E  R  P
     GAGGCCCCCGACCAGACCCTACCCCCCCTTAACAATTTCTCTGTAGCAGAATGCCAGCTGATG  189
 43  E  A  P  D  Q  T  L  P  P  L  N  N  F  S  V  A  E  C  Q  L  M
     AAGACTGAGAGGCCACGACCCAACACCTTTGTCATACGCTGCCTGCAGTGGACCACAGTCATC  252
 64  K  T  E  R  P  R  P  N  T  F  V  I  R  C  L  Q  W  T  T  V  I
     GAGAGGACCTTCCATGTCGACTCTCCAGATGAGAGGGAAGAGTGGATACGGGCTATCCAGATG  315
 85  E  R  T  F  H  V  D  S  P  D  E  R  E  E  W  I  R  A  I  Q  M
     GTAGCCAACAGTCTGAAGCAGCGGGGCCCCGGGGAGGATGCCATGGATTACAAGTGTGGCTCC  378
106  V  A  N  S  L  K  Q  R  G  P  G  E  D  A  M  D  Y  K  C  G  S
     CCCAGTGACTCTTCCACATCTGAGATGATGGAGGTAGCGGTCAGCAAGGCACGGGCCAAAGTG  441
127  P  S  D  S  S  T  S  E  M  M  E  V  A  V  S  K  A  R  A  K  V
     ACCATGAACGACTTCGATTATCTCAAACTCCTCGGCAAGGGCACCTTCGGCAAAGTCATTCTA  504
148  T  M  N  D  F  D  Y  L  K  L  L  G  K  G  T  F  G  K  V  I  L
     GTTCGAGAGAAGGCCACTGGCCGCTATTATGCCATGAAGATCCTGCGGAAGGAGGTCATCATT  567
169  V  R  E  K  A  T  G  R  Y  Y  A  M  K  I  L  R  K  E  V  I  I
     GCAAAGGATGAAGTCGCCCACACAGTCACAGAGAGCCGAGTCCTACAGAATACCAGGCACCCC  630
190  A  K  D  E  V  A  H  T  V  T  E  S  R  V  L  Q  N  T  R  H  P
     TTCCTTACAGCCCTGAAGTATGCCTTCCAGACCCATGACCGCCTGTGCTTTGTGATGGAGTAT  693
211  F  L  T  A  L  K  Y  A  F  Q  T  H  D  R  L  C  F  V  M  E  Y
     GCCAACGGGGGCGACCTGTTTTTCCACCTCTCTCGGGAGCGAGTCTTCACGGAGGATCGGGCA  756
232  A  N  G  G  D  L  F  F  H  L  S  R  E  R  V  F  T  E  D  R  A
     CGCTTTTATGGTGCAGAGATTGTGTCAGCTCTGGAGTACTTGCACTCGACGGATGTGGTGTAC  819
253  R  F  Y  G  A  E  I  V  S  A  L  E  Y  L  H  S  T  D  V  V  Y
     CGCGACATCAAGCTGGAAAACCTTATGCTGGACAAAGATGGCCACATCAAGATCACTGACTTT  882
274  R  D  I  K  L  E  N  L  M  L  D  K  D  G  H  I  K  I  T  D  F
     GGCTTGTCCAAAGAGGGCATCAGTGATGGGGCCACCATGAAAACCTTCTGTGGTACCCCGGAG  945
295  G  L  S  K  E  G  I  S  D  G  A  T  M  K  T  F  C  G  T  P  E
     TACTTGGCGCCTGAGGTGCTAGAGGACAATGACTACGGGCGTGCAGTGGACTGGTGGGGACTG 1008
316  Y  L  A  P  E  V  L  E  D  N  D  Y  G  R  A  V  D  W  W  G  L
     GGCGTGGTCATGTACGAGATGATGTGCGGCCGCCTGCCCTTCTACAACCAGGACCACGAGCGC 1071
337  G  V  V  M  Y  E  M  M  C  G  R  L  P  F  Y  N  Q  D  H  E  R
     CTCTTCGAGCTCATCCTCATGGAGGAGATCCGGTTCCCACGCACGCTTGGGCCAGAGGCCAAG 1134
358  L  F  E  L  I  L  M  E  E  I  R  F  P  R  T  L  G  P  E  A  K
     TCCCTGCTGGCTGGACTGCTCAAGAAGGACCCCAAGCAGAGGCTCGGTGGGGGTCCCAGTGAT 1197
379  S  L  L  A  G  L  L  K  K  D  P  K  Q  R  L  G  G  G  P  S  D
     GCCAAGGAGGTCATGGAGCATCGGTTCTTCCTCAGCATCAACTGGCAGGATGTGGTACAGAAG 1260
400  A  K  E  V  M  E  H  R  F  F  L  S  I  N  W  Q  D  V  V  Q  K
     AAGCTCCTGCCACCCTTCAAACCTCAGGTCACTTCAGAAGTGGACACAAGGTACTTTGATGAT 1323
421  K  L  L  P  P  F  K  P  Q  V  T  S  E  V  D  T  R  Y  F  D  D
     GAATTCACCGCCCAGTCCATCACAATCACACCCCCAGACCGATATGACAGCCTGGGCTCGCTG 1386
442  E  F  T  A  Q  S  I  T  I  T  P  P  D  R  Y  D  S  L  G  S  L
     GAACTGGACCAGCGCACACACTTCCCCCAGTTCTCCTACTCAGCCAGCATCCGAGAGTGAGCA 1449
463  E  L  D  Q  R  T  H  F  P  Q  F  S  Y  S  A  S  I  R  E End
     GCCCTCTGCCACCACAGGACACAGCATGGGCGCCATCCACTGCCTGGGTGGCTTTTTAAAAAA 1512
     AACTTTATTTTGCCTTTGGTTTGCGTTCCCTTCCCTGTCCCCTCATCCCATATCCAGTTTCTT 1575
     CTTCAGTTCTTGCTCAGACTTAGTGGTCCTGGCGGGCCTCTCCTCCGGGCTCCCCTCTGCTCC 1638
     CAGAGGAGTGCTGGGAACCTGCCCAAGGACAGGCCGTTGGGAGGATGGAGAGTGAGGTTATTA 1712
     AT
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Fig. 1 - Continued

study had started. To isolate the full length cDNA clone, a rat testis cDNA library was screened. Partial nucleotide sequence analysis revealed that positive cDNA clones isolated from the library are classified into two groups. As the deduced amino acid sequences of these two groups share extensive homology with those of RAC-PK α and β from other species,

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Rat    α  MNDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYKERPQDVEQRES  50
Mouse  α  ..................................................D....  50
Human  α  .S................................................D...A  50
Rat    β  ..E.SVI.....................S..S........EAPD.TLP  50
Human  β  ..E.SVI.....................S..S........EAPD.TLP  50

Rat    α  PLNNFSVAQCQLMKTERPRPNTFIIRCLQWTTVIERTFHVETPEEREEWT  100
Mouse  α  ..........................................................A  100
Human  α  ..........................................................  100
Rat    β  ........E.............V.................DS.D.....I  100
Human  β  ........E.............V.................DS.D.....M  100

Rat    α  TAIQTVADGLK--RQEEETMDFRSGSPSDNSGAEEMEVALAKPKHRVTMN  148
Mouse  α  ..........|.--.........................S..........  148
Human  α  ..........|.--K....E.................S..........  148
Rat    β  R...M..NS.|.QRGPG.DA..YKC.....S.TS.M....VS.ARAK....  150
Human  β  R...M..NS.|.QRAPG.DP..YKC.....S.TT......VS.ARAK....  150
                   * * *                    ↓

Rat    α  EFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTE  198
Mouse  α  ..........................................................  198
Human  α  ..........................................................  198
Rat    β  D.D..............R.............R....I........V..  200
Human  β  D.D..............R.............R....I........V..  200

Rat    α  NRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSED  248
Mouse  α  ..........................................................  248
Human  α  ..........................................................  248
Rat    β  S.....T..........A.................D...........T..  250
Human  β  S.....T..........A.............................T.E  250

Rat    α  RARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKE  298
Mouse  α  ..........................................................  298
Human  α  ..........................................................  298
Rat    β  ............E....-TD.....I.....................S..  299
Human  β  ............E....-RD.....I.....................  299

Rat    α  GIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLP  348
Mouse  α  ..........................................................  348
Human  α  ..........................................................  348
Rat    β  ..S.....................................................  349
Human  β  ..S.....................................................  349

Rat    α  FYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPTQRLGGGSED  398
Mouse  α  ..........................................................  398
Human  α  ..........................................K.........  398
Rat    β  .......R.....................A.......K......PS.  399
Human  β  .......R.............S.....A.......K......PS.  399

Rat    α  AKEIMQHRFFANIVWQDVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMIT  448
Mouse  α  ..........................................................  448
Human  α  ..........G....H..........................................  448
Rat    β  ...V.E....LS.N....VQ...L..........V......D.....S..  449
Human  β  ...V.E....LS.N....VQ...L..........V......D.....S..  449

Rat    α  ITPPDQDDSMECVDSERRPHFPQFSYSASGTA                    480
Mouse  α  ..............................                    480
Human  α  ..............................S..                 480
Rat    β  .....RY..LGSLELDQ.T..........IRE                  481
Human  β  .....RY..LGLLELDQ.T..........IRE                  481
Human  β' .....RY..LGLLELDQ.T.........FREEKDLLMSLFVSLILFSDFS  499

Human  β' SLKSHSFSSNFILLSFSSLKK                              520
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Fig. 2. Comparison of the amino acid sequences of the RAC-PK from different species. Gaps (–) have been introduced for optimal alignment. Dots (·) show identities with the rat RAC-PK α. The PH domain sequences are boxed. The conserved amino acid residues in ATP-binding consensus sequences, Gly-157, Gly-159, Gly-162, and Lys-179 for the rat rac-PK α are emphasized by asterisks and an arrow. Shown are sequences for rat α (this report), mouse α (7), human α (3), rat β (this report), and human β (9). The carboxyl-terminal amino acid sequence 450 to 520 of the human RAC-PK β (8) is shown as human β'.

respectively (3-9), these two groups were concluded to encode the rat homologues of RAC-PK α and β. The nucleotide and deduced amino acid sequences are shown in Fig. 1.

Fig. 2 shows the alignment of the predicted amino acid sequences of RAC-PK α and β from different species. The rat RAC-PK α has sequence identity of 99.4% and 97.9% with the mouse (7) and the human RAC-PK α (3), respectively. Concerning with RAC-PK β, the clones have been isolated by two groups independently from human libraries (8,9). Amino acid sequences 1 to 477 are identical between these two human RAC-PK β clones, however, the clone isolated by Jones et al. (8) has a carboxyl-terminal extension of 40 amino acids in comparison with the clone isolated by Cheng et al. (9). It is not clear why these two clones have different 3' end sequences. None of the rat RAC-PK β clones obtained in this study has such a carboxyl-terminal extension, and the rat RAC-PK β is 97.7 % identical to the human RAC-PK β (9). The carboxyl-terminal protein kinase catalytic domains (the amino acid sequence 150 to 408 and 152 to 409 of the RAC-PK α and RAC-PK β, respectively) closely related PKC and PKA as described (3-9). The putative regulatory region termed PH domain (the boxed region in Fig. 2) is also conserved well among the RAC-PK family.

The expression of RAC-PK α and β has been studied using human cell lines (3,8,9) and tissues (5,7) for each subtype by RNA blot analysis. Fig. 3 shows the comparison of the expression of the two rat RAC-PK in various rat tissues. The 3.2 kb band of RAC-PK α transcript and the 3.4 kb band of RAC-PK β transcript were detected in all tissues examined, but the level of expression was different each other. For example, the expression of RAC-PK α is much higher than that of RAC-PK β in brain and lung, whereas RAC-PK β is highly expressed in testis but RAC-PK α is very low in this tissue. Distinct expression of the RAC-PK family may reflect different function of each subtype.

It has been shown that v-*akt* oncogene is generated by fusion of the viral *Gag* and the cellular c-*akt* (RAC-PK) sequences (22). As modification of the amino-terminal region is essential for v-*akt* to gain the transforming activity, it seems that the amino-terminal region including the PH domain is important for the regulation of its protein kinase activity.

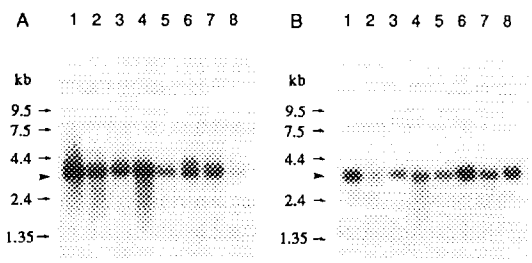

Fig. 3. RNA blot analysis in rat tissues. A, RAC-PK α. B, RAC-PK β. Poly $(A)^+$ RNA (2 μg) isolated from various rat tissues was analyzed with each DNA probe labeled with [α-^{32}P]dCTP as described in "EXPERIMENTAL PROCEDURES." The signals generated with the β-actin probe as a control were approximately the same among the tissues. The positions of size markers are indicated in kb. The positions of the transcript of RAC-PK α (3.2 kb) and RAC-PK β (3.4 kb) are shown by arrows. Lanes 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis.

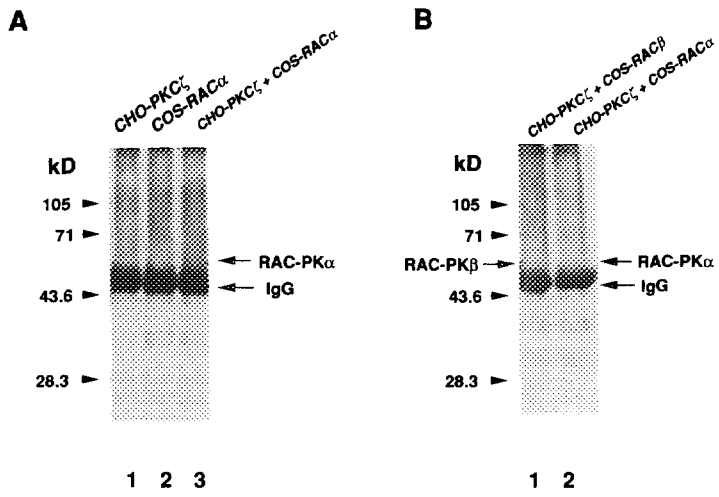

Fig. 4. Co-immunoprecipitation. Immunoprecipitation was carried out using the antiserum against PKC ζ, and immunoblot analysis was done by using the antibody against RAC-PK as described in "EXPERIMENTAL PROCEDURES." A, Co-immunoprecipitation of the RAC-PK α with PKC ζ. Lane 1; the extract of CHO cells overexpressing PKC ζ (CHO-PKC ζ), alone. Lane 2; the extract of COS-7 cells transfected with the plasmid of the RAC-PK α (COS-RACα), alone. Lane 3; the mixture of CHO-PKC ζ and COS-RAC α. B. Co-immunoprecipitation of RAC-PK α and β with PKC ζ. Lane 1; the mixture of CHO-PKC ζ and the extract of COS-7 cells transfected with the plasmid of RAC-PK β (COS-RAC β). Lane 2; the mixture of CHO-PKC ζ and COS-RAC α. The protein bands of RAC-PK α, RAC-PK β, and heavy chains of immunoglobulin are indicated by arrows. The positions of size markers are indicated in kD.

Recently, PH domains derived from various proteins were indicated to bind to the βγ subunits of the heterotrimeric GTP binding protein (23,24). Thus, a possibility has risen that the PH domain of the RAC-PK may associate with some proteins such as the βγ subunits to regulate its protein kinase activity. We found that some proteins bind to the PH domain of RAC-PK and one of these binding proteins is PKC ζ by using a fusion protein of glutathion-S-transferase and the PH domain of RAC-PK (Konishi, H., Kuroda, S., and Kikkawa, U., manuscript in preparation). Fig. 4 shows co-immunoprecipitation of RAC-PK and PKC ζ. After incubation of RAC-PK α expressed in COS-7 cells and PKC ζ extracted from CHO cells, immunoprecipitation was carried out using the polyclonal antibody specific to PKC ζ. A protein band was detected in the immunoprecipitate by using the antiserum against RAC-PK (Fig. 4A, lane 3), and the protein band was not detected in the immunoprecipitate from COS-7 cells or from CHO cells (Fig. 4A, lanes 1 and 2). The position of the immunoreactive protein band was identical to that of RAC-PK α partially purified from the transfected COS-7 cells, and PKC ζ was found in these immune-complexes by the antibody against PKC ζ (data not shown). These results indicate that RAC-PK α associates with PKC ζ in crude extracts. RAC-PK β was detected in the immunoprecipitate when incubated with PKC ζ and then precipitated with the antiserum against PKC ζ (Fig. 4B). Very recently, Bruton tyrosine kinase was reported to associate with PKC subspecies through its PH domain (25). Therefore, the PH domain of several proteins may interact with PKC subspecies. It is

necessary to study further the mechanism and role of the association of RAC-PK and PKC through the PH domain.

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