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Molecular cloning of a second form of *rac* protein kinase

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A novel serine/threonine protein kinase (termed *rac*-PK) has recently been identified and cloned from cDNA libraries derived from the human cell lines MCF-7 and WI38. A second form of this protein kinase, termed *rac* protein kinase β, has been identified from cDNAs derived from the same cell lines. These two closely related forms show 90% homology, although the β form with a predicted M_r 60 200 has a carboxyl terminal extension of 40 amino acids in comparison to the α form. This extension has a high serine content with 11 serine residues in the last 30 amino acids. The β form of the protein has been shown by both in vitro translation and bacterial expression to be ~5000 Da larger than the α form. *rac* protein kinase β is encoded by a 3.4-kb transcript and the α form is encoded by a 3.2-kb mRNA. Using gene-specific probes both transcripts were detected in all cell types analyzed, although levels of expression were different for the two forms. The catalytic domain of *rac* protein kinase β shows a high degree of homology to both the protein kinase C and cyclic AMP-dependent protein kinase families, and hence *rac* protein kinases appear to represent a new subfamily of the second messenger serine/threonine protein kinases.

Introduction

Despite considerable biochemical scrutiny, the complexity of protein kinases has only been appreciated through the advent of molecular biological techniques. The conserved nature of the protein kinase catalytic domain (reviewed by Hanks *et al.,* 1988; Hunter and Cooper, 1986) has allowed the identification of both homologous and more distantly related protein kinases (Hanks, 1987) not yet identified on the basis of activity. Through this approach, many protein kinases have been shown to exist not only as a single enzyme but as groups of closely related proteins. The best-characterized examples of such families are protein kinase C (PKC)[1] (reviewed by Nishizuka, 1988: see also Osada *et al.,* 1990; Bacher *et al.,* 1991) and cyclic AMP (cAMP)-dependent protein kinase (cAMP-PK) (reviewed by Edelman *et al.,* 1987; Hanks *et al.,* 1988; see also Beebe *et al.,* 1990). Subfamilies have now been identified for both serine/threonine- and tyrosine-specific protein kinases, including *src, abl,* insulin receptor, calcium-calmodulin-dependent protein kinase, and *cdc*2/CDC28 protein kinases (reviewed by Hanks, 1991). The existence of isoforms of a protein kinase allows diversification in the form of tissue-specific expression and biochemical properties including substrate specificity, kinetics, and mode of activation, which all give greater flexibility to a biological system.

Recently, we reported (Jones *et al.,* 1991) the identification and cloning of a new member of the serine/threonine second messenger subfamily of protein kinases, termed *rac* protein kinase (*r*elated to *A* and *C* protein kinases; *rac*-PK). Here we describe the cloning of a protein kinase closely related to *rac*-PK that also shows a high degree of homology to both PKC and cAMP-PK subfamilies.

Results

cDNA library screening and molecular cloning

During the identification and purification of cDNA clones encoding *rac*-PK (Jones *et al.,* 1991), two additional cDNAs were identified in a cDNA library derived from WI38 lung fibroblasts. Further analysis of these clones (λWI38*rac* 26 and λWI38*rac* 28) revealed the existence of a second form of the *rac* protein kinase. λWI38*rac* 26 and λWI38*rac* 28 (see Figure 1A) overlapped to give ~1-kb coding se-

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[1] Abbreviations used: bp, base pair(s); cAMP-PK, cyclic AMP-dependent protein kinase; PKC, protein kinase C; *rac*-PK, *rac* protein kinase; SDS, sodium dodecyl sulphate.

quence of a protein that was closely related, but not identical, to the original *rac*-PK clone. The previously described sequence (Jones *et al.*, 1991) has been termed *rac*-PKα, and this second form has been termed *rac*-PKβ. cDNAs previously isolated during the screening of various cDNA libraries for *rac*-PKα were rescreened using the 160 base pair (bp) *Eco*RI-*Nco* I fragment from the 5′ end λWI38*rac* 26, which resulted in the isolation of a full-length coding clone, λMCF*rac* 2F1/1, from a cDNA library derived from MCF-7 cells.

Sequence of human rac protein kinase β

The complete sequence of λMCF*rac* 2F1/1 was determined on both DNA strands using the strategy shown in Figure 1A. The 1851 bp cDNA sequence, shown in Figure 1B, contained a single open reading frame from nucleotides 205–1764. The clone contains 204 bp of 5′ untranslated region and but only 88 bp of 3′ noncoding region. The lack of both polyadenylylation signal and polyadenylylated tail indicated that this clone did not represent the complete mRNA. The predicted initiator methionine was in a favorable context for translation initiation (Kozak, 1984) with the sequence CCACCATG. The predicted open reading frame of 1560 bp encodes a protein of 520 amino acids with M_r 60 200.

The predicted protein sequence of *rac*-PKβ contains a classic protein kinase domain between amino acid residues 151 and 412. The sequence motif -Gly-X-Gly-X-X-Gly- (positions 159–164) with the conserved Lys (position 181) conforms to the consensus ATP-binding motif (Wierenga and Hol, 1983). The two motifs -Asp-Ile-Lys-Leu-Glu-Asn- (positions 275–280) and -Gly-Thr-Pro-Glu-Tyr-Leu-Ala-Pro-Glu- (positions 312–320) confer serine/threonine rather than tyrosine specificity (Hanks, 1987). Another highly conserved group of amino acids is -Asp-Phe-Gly- (positions 293–295). *Rac*-PKβ (as with *rac*-PKα) contains all the residues that have been shown to be virtually invariant between protein kinases (Hanks *et al.*, 1988). Both *rac*-PKs also contain three glutamate residues (yeast cAMP-PK C_1, E171, E214, and E274; *rac*-PKα, E234, E278, and E341) that have recently been implicated in substrate recognition (Gibbs and Zoller, 1991). Important residues of cAMP-PK involved in substrate recognition, nucleotide binding, and catalysis have been delineated by biochemical methods and more recently by solution of the crystal structure of murine cAMP-PK Cα (Knighton *et al.*, 1991a,b). Of these residues, all but six are conserved in *rac*-PKα with

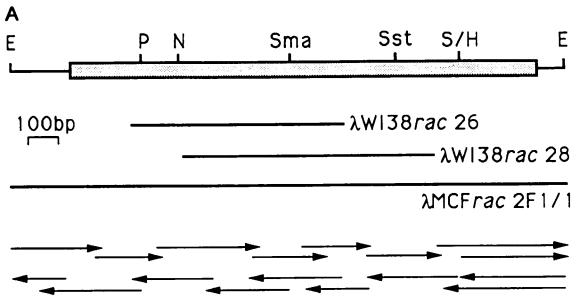

Figure 1. **Restriction map, cDNA clones, and sequencing strategy for *rac*-PKβ.** (A) Restriction sites used for sequencing are shown on the schematic representation of the cDNA structure encoding *rac*-PKβ. The coding region of the cDNA (boxed region) is flanked by 5′ and 3′ untranslated regions (solid bars). The restriction enzymes used for this analysis were *Eco*RI (E), *Pst* I (P), *Nco* I (N), *Sma* I (Sma), *Sst* I (Sst), *Sal* I (S), and *Hinc* II (H). cDNAs encoding *rac*-PKβ are shown below. Arrows indicate the overlapping sequence data obtained for both strands of the clone λMCF*rac* 2F1/1. (B) Nucleotide sequence and predicted amino acid sequence of *rac*-PKβ. The amino acid sequence, numbered 1–520, is shown below the respective codons. Asterisks denote in-frame stop codons.

substitutions at Lys158 (Thr 51), Leu278 (Pro169), Leu295 (Phe187), Phe309 (Leu198), Glu440 (Asp329), and Phe442 (Glu331). In addition to the changes noted for the α isoform, *rac*-PKβ has a further conservative substitution at Ile276 (Leu167). Interestingly, Thr197, phosphorylated in cAMP-PK Cα, is conserved in *rac*-PKα and β (Thr308 in *rac*-PKα) as are the residues identified as preventing dephosphorylation of this residue (His194, Arg273, Lys297, and Thr305 in *rac*-PKα). It should be noted, however, that of the residues involved in cAMP-PK Cα kinase function, many are also conserved in the PKCs.

Alignment of rac protein kinases

The predicted amino acid sequences of human *rac*-PKα and β are shown aligned in Figure 2. Overall, the two proteins show 90% homology (81% identity), with the highest degree of homology in the catalytic domains (97% homology). The amino terminal domains show 86% homology, and the carboxyl termini are the most divergent with 80% homology. Interestingly, *rac*-PKβ has a 40 amino acid extension containing 12 serine residues at the carboxyl terminus in comparison to *rac*-PKα. This carboxyl-terminal 40 amino acids also contains three repeated motifs of SDFSSLK (residues 496–502), SFSSNF (residues 505–510), and SFSSLK (residues 514–519), although database searches revealed no significant homologies. Within the

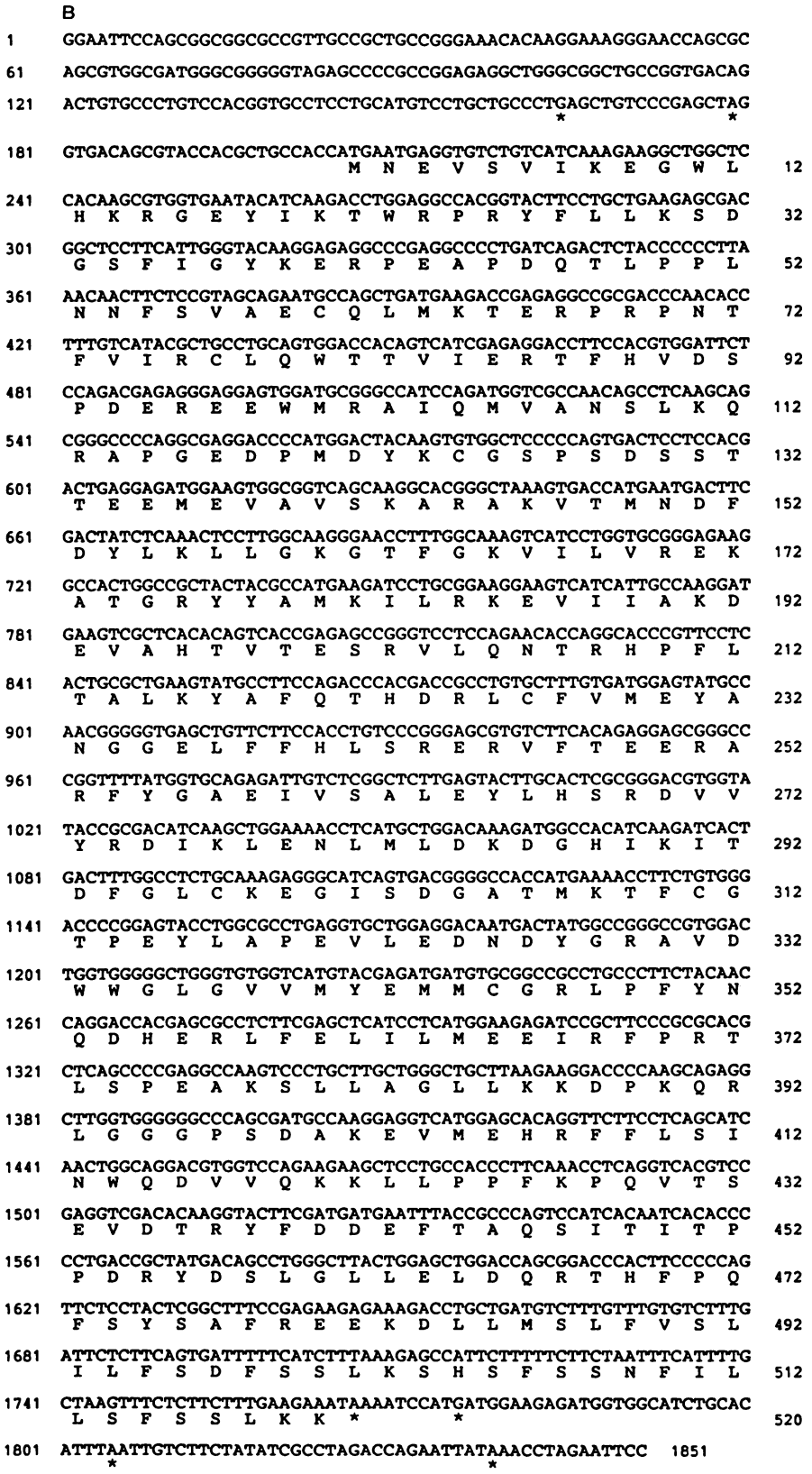

Figure 1. **(Continued)**

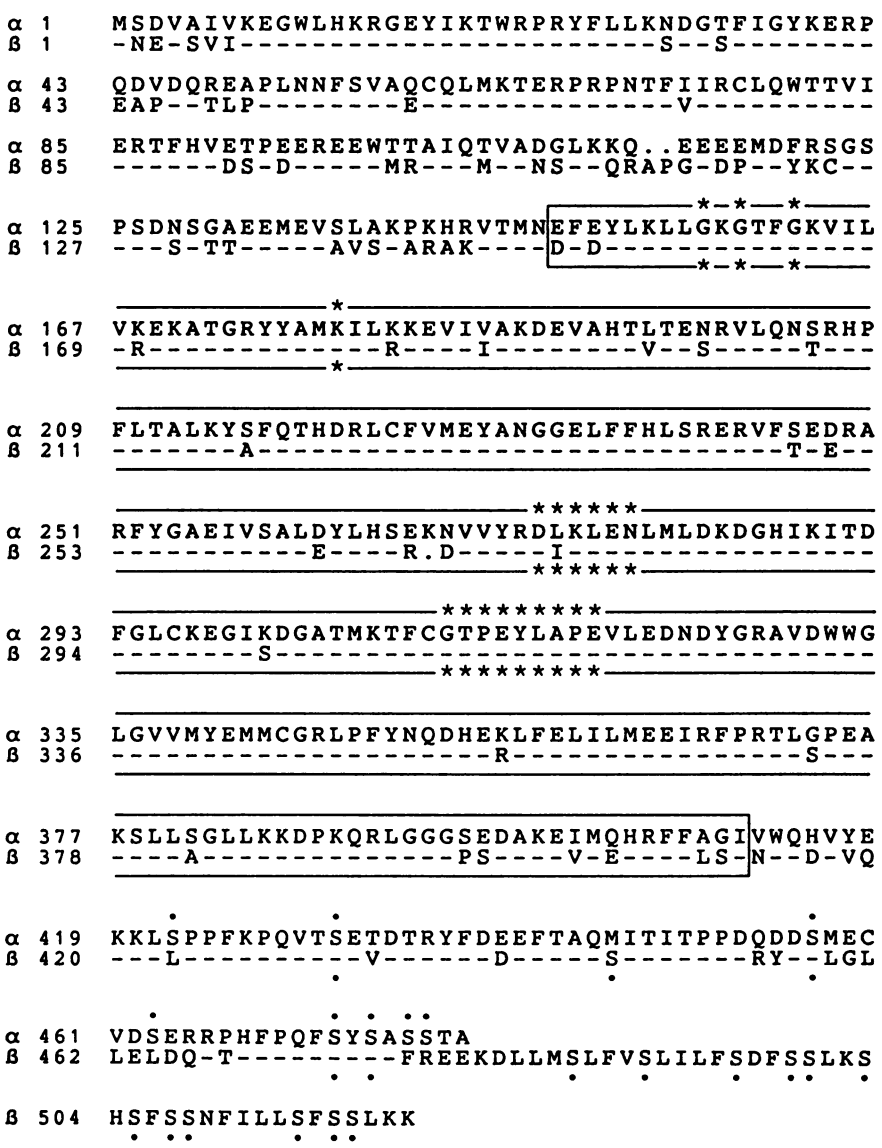

Figure 2. **Alignment of the predicted peptide sequences of *rac*-PKα and β.** The two sequences were aligned by eye; the β peptide sequence is shown only as differences from the α sequence and conserved amino acids are denoted by (-). Gaps introduced into either sequence for maximum alignment are denoted by (·). The boxed region indicates the catalytic domain (1) with asterisks indicating some residues characteristic of serine/threonine protein kinases. Heavy dots (•) highlight serine residues in the carboxyl terminal domains of both proteins.

catalytic domain of 265 residues, there are only 25 amino acid changes, of which 20 are conservative substitutions.

Confirmation of predicted open reading frame

In vitro expression in a cell-free reticulocyte translation system was used to analyze the molecular weight of the protein encoded by the clone λMCF*rac* 2F1/1. Sense and antisense transcripts were synthesized in vitro from cDNA constructs under the control of the T7 or T3 RNA polymerase promoters. In the presence of *rac*-PKβ sense transcript, a major polypeptide with an apparent M_r of 63 000 was synthesized (Figure 3, lane 3), whereas in the presence of *rac*-PKα sense transcript a polypeptide of apparent M_r 58 000 was synthesized (Figure 3, lane 4). These apparent molecular weights both correlated well with the predicted M_r of 60 200 and 55 700, respectively. In the absence of exogenous RNA (Figure 3, lane 1) or in the presence of *rac*-PKβ antisense transcript (Figure 3, lane 2) no major polypeptides were synthesized. The molecular weight of the protein encoded by λMCF*rac* 2F1/1 was further analyzed by expression in an inducible bacterial system. cDNAs encoding both *rac*-PKα and β were inserted into the expression vector pRK172 (McLeod *et al.*, 1987) via an engineered *Nde* I restriction site at the presumed initiator codon.

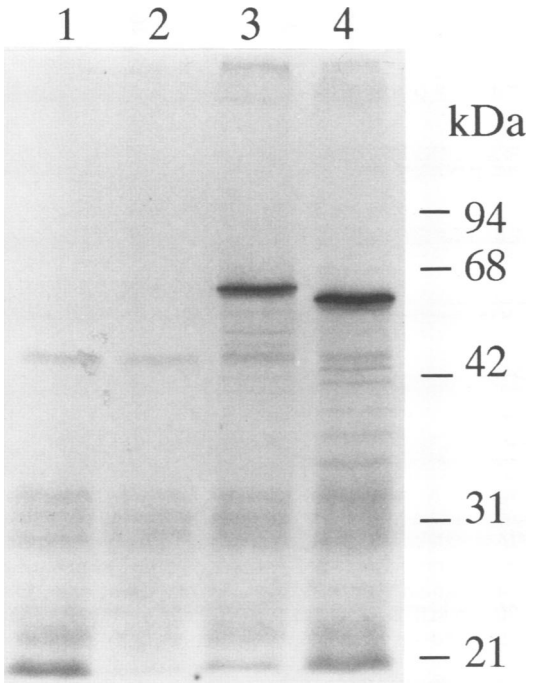

Figure 3. **In vitro translation of *rac*-PKα and β.** Sense and antisense transcripts were translated in a cell-free rabbit reticulocyte lysate in the presence of [^{35}S]methionine as described in text. Translation products were analyzed by 10% SDS/polyacrylamide gel electrophoresis followed by fluorography at −70°C overnight with two intensifying screens. Lanes: 1, no exogenous RNA; 2, antisense *rac*-PKβ transcript; 3, sense *rac*-PKβ transcript; 4, sense *rac*-PKα transcript. Molecular size markers expressed in kDa are shown.

Expression from *rac*-PKβ cDNA-generated inclusion bodies containing a major polypeptide M_r 63 000, whereas the molecular weight of the protein synthesized from *rac*-PKα cDNA was 58 000 (data not shown). Hence, we conclude that the predicted open reading frame of *rac*-PKβ is correct.

Homology between rac protein kinases and other protein kinases

Database searches using the predicted *rac*-PKβ peptide show the same pattern of homologies as seen with the α form. The most homologous protein kinases are murine PKCη (77% homology and 51% identity in catalytic domain) and bovine cAMP-PKβ catalytic subunit (66% homology and 46% identity, respectively). The program PILEUP (University of Wisconsin Genetics Computer Group software package 7.0β) was used to calculate the pairwise alignments between various protein kinase catalytic domains (Feng and Doolittle, 1987) using the algorithm of Needleman and Wunsch (1970). The protein kinases chosen were the PKC family, the cAMP-PK family, and both the S6 kinase (70 kDa) and the cyclic GMP-dependent protein kinase because these are also members of the second messenger subfamily. These relationships are depicted in Figure 4. Although this is not a phylogenetic reconstruction, the dendrogram may be used to deduce evolutionary branch points. This showed that *rac* protein kinases probably diverged from the PKC family after the divergence of PKC and cAMP-PK. The alignments produced by the program PILEUP were then taken and used pairwise to calculate the homologies between the different kinase domains, using the program GAP. Table 1 lists the homologies between the catalytic domains of *rac*-PK-α and β, and members of the PKC, and cAMP-PK families. This shows the similarity

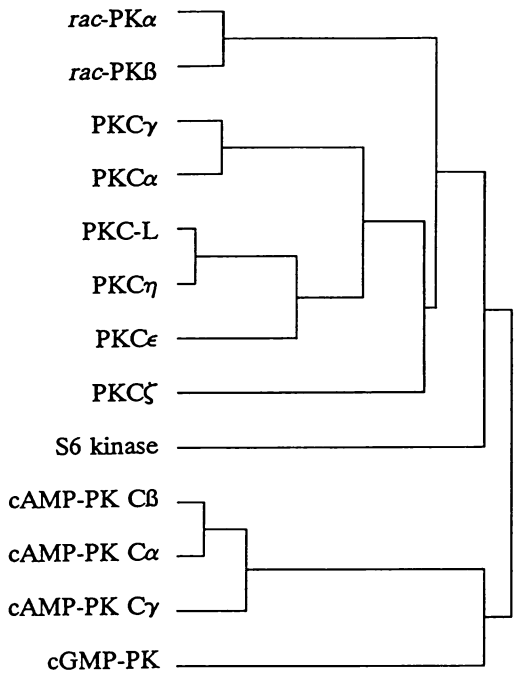

Figure 4. **Pairwise alignment relationships between the catalytic domains of protein kinases of the second messenger subfamily.** The catalytic domains as defined by Hanks *et al.* (1988) of human *rac*-PKα (Jones *et al.*, 1991), human *rac*-PKβ, rabbit PKCα (Ohno *et al.*, 1987), rabbit PKCγ (Ohno *et al.*, 1987), rat PKCε (Ono *et al.*, 1988), rat PKCζ (Ono *et al.*, 1988), murine PKCη (Osada *et al.*, 1990), murine PKC-L (Bacher *et al.*, 1991), bovine cAMP-PK Cα (Shoji *et al.*, 1983), bovine cAMP-PK Cβ (Showers and Maurer, 1986), human cAMP-PK Cγ (Beebe *et al.*, 1990), bovine cGMP-dependent protein kinase (cGMP-PK) (Takio *et al.*, 1984), and rat S6 kinase (Kozma *et al.*, 1990) were used in a pairwise progressive alignment (Feinberg and Doolittle, 1987) using the algorithm of Needleman and Wunsch (1970) (PILEUP, GCG version 7.0β). The dendrogram represents these pairwise alignments where the horizontal distance is a measure of the relatedness of the sequences.

between *rac*-PKα and β in their relation to both PKC and cAMP-PK families.

Northern analysis

The levels of expression of *rac*-PKα and *rac*-PKβ were analyzed in various human cell lines using probes derived from the 3′ termini of both cDNAs. The α-specific probe, a 370-bp *Sty* I-*Eco*RI fragment (nucleotides 1282–1652) from the full-length coding cDNA clone λWI38*rac* 71, contained 240 bp encoding the carboxyl terminal 80 amino acids and 129 bp of 3′ untranslated region. The β-specific probe, a 349-bp *Hinc* II-*Eco*RI fragment (nucleotides 1502–1851) from the cDNA clone λMCF*rac* 2F1-1, contained 261 bp encoding the carboxyl terminal 87 amino acids and 89 bp 3′ untranslated region. The α probe hybridized to a 3.2-kb transcript that is expressed in all the cell types analyzed (Figure 5) whereas the β probe hybridized to a 3.4-kb transcript also present in all cell types. A small degree of cross hybridization can be seen between the α transcript and the β probe. The striking difference between the two transcripts is the variation in the level of expression. The level of expression of the α transcript was ~100-fold higher in MCF-7 cells than in A1146 cells whereas the β transcript showed only ~15-fold variation in level of expression between MCF-7 cells and both A1146 and T47D cells.

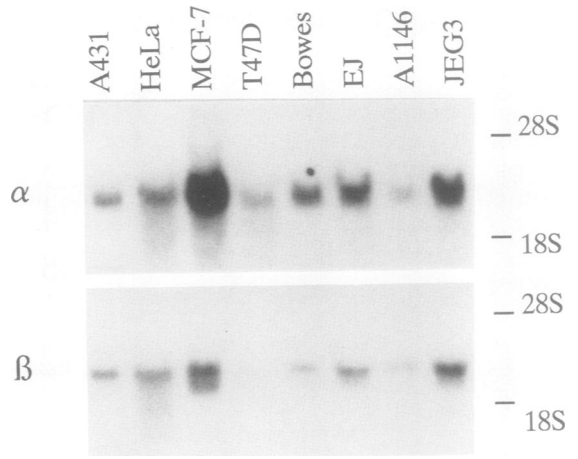

***Figure 5.* Northern blot analysis of *rac*-PKα and β expression in various human cell lines.** Northern blots of total RNA isolated from various human cell lines were hybridized to cDNA probes specific for the carboxyl termini of both *rac*-PKα and *rac*-PKβ (see text). Washing was at 65°C for 3 × 30 min in 0.1× SSC, 0.1% SDS. Positions of 28S and 18S rRNA bands are shown.

Discussion

Recently, we reported the molecular cloning of a new member of the serine/threonine protein kinase family, termed *rac*-PK. Here, we describe the identification of a closely related protein, termed *rac*-PKβ. cDNA clones encoding the complete open reading frame have been identified and the sequence determined. The degree of homology between the catalytic domains of these two proteins is 97%, indicating that the two kinases have a similar, if not identical, function. The most divergent regions of the two forms are found at the carboxyl termini. *rac*-PKβ has a 40 amino acid extension containing 12 serine residues. *rac*-PKα also has a high serine content at the carboxyl terminus, suggesting that phosphorylation may be involved in regulation of activity as seen for example with the

Table 1. Homologies between the kinase domains of *rac*-PKα and *rac*-PKβ and both PKC and cAMP-PK families

	rac-PKα		*rac*-PKβ	
	Percentage similarity	Percentage identity	Percentage similarity	Percentage identity
PKCα	72.5	50.4	72.1	50.0
PKCγ	71.4	49.6	71.4	51.1
PKCϵ	74.8	52.3	74.0	50.8
PKCζ	74.8	49.6	75.2	51.1
PKCη	77.9	53.4	77.1	51.5
PKC-L	76.3	51.9	75.6	50.4
cAMP-PK Cα	67.2	46.3	65.6	44.0
cAMP-PK Cβ	67.6	47.1	66.4	46.0
cAMP-PK Cγ	66.0	44.8	64.5	42.9

Percentage homology and identity between catalytic domains of *rac*-PKs and other protein kinases of the second messenger subfamily. The alignments of the catalytic domains of *rac*-PKα, *rac*-PKβ, PKCα, PKCγ, PKCϵ, PKCζ, PKCη, PKC-L, cAMP-PK Cα, cAMP-PK Cβ, and cAMP-PK Cγ were produced by the program PILEUP (GCG) and used in pairwise GAP (GCG) alignments to calculate the degree of similarity and identity between the different kinases. The sequences used in this analysis are the same as those in Figure 4.

S6 kinase (Ballou *et al.,* 1988). Another interesting feature of the β protein is the conservation of one of the two negatively charged regions in *rac*-PKα (residues 94–98 and 114–117). It is possible that these regions are involved in charge compensation through interaction with another protein or even with another subunit.

As with *rac*-PKα, *rac*-PKβ shows the highest homology to PKCη and the cAMP-PK β catalytic subunit. Although the degree of homology to PKCη and PKC-L within the catalytic domain is high, *rac*-PKs appear not to be members of the PKC family for the following reasons: 1) PKCs contain two common Cys-rich sequences in the regulatory region of the protein (although PKCζ has only one of these sequences), which is lacking in both of the predicted *rac*-PK peptide sequences and 2) PKCs have a pseudosubstrate sequence in the amino terminal domain (-X-X-Arg-Ala-Leu-X-X- where X is a basic amino acid) (House and Kemp, 1987), which is not found in either of the *rac*-PK-predicted peptide sequences. Similarly, it appears unlikely that *rac*-PKs represent further isoforms of the cAMP-PK family because: 1) *rac*-PKs have 148 and 149 amino acids in the region to the amino terminal of the catalytic domain whereas the cAMP-PKs have ~42 amino acids in this region and 2) The degree of homology between cAMP-PK catalytic subunit isotypes is considerably higher (96–97%) than that seen between *rac*-PKs and this family (64–67%). Both the cAMP-PK and PKC subfamilies are involved in separate second messenger-mediated signal transduction pathways. It is possible that *rac*-PKα and β represent another family of protein kinases also involved in signal transduction. Elucidation of signal transduction pathways that modulate the activity of *rac*-PKs may shed light on their function in vivo.

Both *rac*-PKα and *rac*-PKβ are expressed in all the human cell types so far analyzed. The level of expression of the β form is, in general, lower than that of the α transcript with lower variation in the level of expression seen with the β than seen with the α transcript. Hence, it would appear that the two genes have different modes of regulation, possibly through different promoter structures or different mRNA stabilities. The isolation of the promoters of these two genes may elucidate their different modes of transcriptional regulation. Furthermore, analysis of *rac*-PKα and β expression in a wider range of cell types may show cell-specific elevated expression of the β form.

The development of specific antisera will allow the identification of the kinase activity associated with *rac*-PKβ and possibly allow purification. Characterization of *rac*-PKα activity in either immunoprecipitates or after immunoaffinity purification (Jakubowicz, Jones, and Jennings, unpublished data) showed that it is a general protein kinase, capable of phosphorylating several known in vivo phosphoproteins, such as myelin basic protein, glycogen synthetase, regulatory subunit II of cAMP-PK, histone H1, 40S ribosomal subunit, and microtubule-associated protein 2 (Jones *et al.,* 1991). Analysis of *rac*-PKα with peptide substrates indicated a PKC-like activity in that it required basic residues downstream of the target serine residue for optimal activity (Jakubowicz, Jones, and Jennings, unpublished data). Given the similarity of the catalytic domains of *rac*-PKα and β, it is probable that their target proteins in vivo are similar. However, the difference in the carboxy terminal domains suggests that these two isoforms may be differentially regulated.

Further experiments directed toward identifying the mechanism of activation/inactivation of *rac*-PK in vivo and the second messenger system involved in this putative regulation will provide insights into its function in vivo and general significance. Recent experiments have indicated that the *Drosophila* homolog of *rac*-PK is highly conserved, encoded by a single gene that is both maternally and zygotically regulated, and is expressed throughout development (Jones, Bilbe, Grossniklaus, and Hemmings, unpublished data). The powerful genetic approaches offered by *Drosophila* for the analysis of gene function will help elucidate the role of *rac*-PK in signal transduction.

Materials and methods

Molecular cloning

Human cDNA libraries derived from MCF-7 epithelial cells (P. Chambon, INSERM, Strasbourg, France) or WI38 fibroblasts (Clontech, Palo Alto, CA and Stratagene, La Jolla, CA) were screened as previously described (Stone *et al.,* 1987). Filters were prehybridized in 6× SSC (1× SSC contains 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5× Denhardts (1× Denhardts contains 0.02% [wt/vol] Ficoll, 0.02% [wt/vol] bovine serum albumin, 0.02% [wt/vol] polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate (SDS), 0.05% sodium pyrophosphate, and 0.25 mg/ml DNA at 60°C and hybridized overnight to a cDNA probe (1—2 × 10^6 cpm/filter) (Feinberg and Vogelstein, 1983) in the same solution. Filters were washed at 60°C in 1× SSC, 0.1% SDS for 2 h. Positive clones were plaque purified and the *Eco*RI inserts subcloned for further analysis.

DNA sequencing

cDNAs were sequenced by the dideoxy nucleotide chain termination method (Sanger *et al.,* 1977) using Sequenase

(USB, Cleveland, OH) according to the manufacturer's protocols. Sequencing reactions were primed with either the universal or reverse sequencing primers or specific oligonucleotides. 7-deaza-dGTP was utilized to overcome difficult G + C-rich regions. DNA sequence analysis was performed using the University of Wisconsin Genetics Computer Group software package (Devereux *et al.,* 1984), version 7.0β.

Northern analysis

Total RNA was isolated from cells using the method of Chomczynski and Sacchi (1987). For Northern analysis, 20 μg of total RNA was fractionated on a 1% formaldehyde/agarose gel and transferred to a nylon membrane (Zeta-probe, Bio-Rad, Richmond, CA) as previously described (Khew-Goodall and Hemmings, 1988). Prehybridization was at 42°C in a solution containing 50% formamide, 5× SSC, 5× Denhardts, 2% SDS, 0.5% sodium pyrophosphate, and 0.25 mg/ml DNA. Hybridization to 2×10^7 cpm of radiolabeled cDNA probe (specific activity $\sim 10^9$ cpm/μg) was at 42°C overnight. Washing was at 65°C for 3 × 30 min in 0.1× SSC, 0.1% SDS. Autoradiography was at −70°C with two intensifying screens. Preflashed autoradiographs were quantitated by densiometry.

In vitro transcription and translation

*Eco*RI inserts from λWI38*rac* 71 (Jones *et al.,* 1991) and λMCF*rac* 2F1/1 were subcloned in both orientations into pBluescript (Stratagene, La Jolla, CA) and linear templates were used for in vitro transcription using either T7 or T3 RNA polymerase. The capped RNA (Nielsen and Shapiro, 1986) was translated in a cell-free rabbit reticulocyte lysate in the presence of [^{35}S]methionine at 30°C for 1 h according to the manufacturer's recommendations (Stratagene). Samples were subjected to 10% SDS/polyacrylamide gel electrophoresis (Laemmli, 1970) prepared for fluorography by soaking in 1 M sodium salicylate for 15 min (Chamberlain, 1979) and then dried. Fluorography was overnight at −70°C with two intensifying screens.

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Note added in proof. Nucleotide sequence accession number. *rac*-PKβ sequence has been deposited in the Genebank database under accession number M77198 and M63167 for the α sequence.

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