*Proc. Natl. Acad. Sci. USA*
Vol. 88, pp. 4171–4175, May 1991
Biochemistry

# Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily

**(protein phosphorylation/signal transduction/homology to protein kinase A and protein kinase C/MCF-7 and WI38 cells)**

PAMELA F. JONES, TERESA JAKUBOWICZ, FERNANDO J. PITOSSI*, FRANSISCA MAURER, AND BRIAN A. HEMMINGS†

Friedrich Miescher-Institut, Postfach 2543, CH-4002 Basel, Switzerland

*Communicated by Martha Vaughan, February 11, 1991*

**ABSTRACT** **A partial cDNA was isolated that encoded a protein kinase, termed rac (related to the A and C kinases). This cDNA was subsequently used to screen libraries derived from the human cell lines MCF-7 and WI38 and led to the isolation of full-length cDNA clones. DNA sequence analysis identified an open reading frame of 1440 base pairs encoding a protein of 480 amino acids ($M_r$, 55,716). This result was supported by the synthesis of a $M_r$ 58,000 protein in an *in vitro* translation system that used RNA transcribed from cloned cDNAs with SP6 RNA polymerase. The predicted protein contains consensus sequences characteristic of a protein kinase catalytic domain and shows 73% and 68% similarity to protein kinase C and the cAMP-dependent protein kinase, respectively. Northern (RNA) analysis revealed a single mRNA transcript of 3.2 kilobases that varied up to 300-fold between different cell lines. Specific antisera directed towards the carboxyl terminal of the rac protein kinase were prepared and used to identify a protein of $M_r$ 59,000 by immunoblotting. A specific protein kinase activity was identified that phosphorylated several substrates in immunoprecipitates prepared with the rac-specific antisera.**

Protein phosphorylation is a fundamental process for the regulation of cellular functions. It is the coordinated action of both protein kinases and phosphatases that controls the phosphorylation levels and, hence, the activity of specific target proteins (for review, see refs. 1 and 2). One of the predominant roles of protein phosphorylation is in signal transduction, where extracellular signals are amplified and propagated by a cascade of protein phosphorylation and dephosphorylation events. Two of the best-characterized signal transduction pathways involve the cAMP-dependent protein kinase (cAMP-PK) and protein kinase C (PKC) (1). Each pathway uses a different second-messenger molecule to activate the protein kinase, which, in turn, phosphorylates specific target molecules. Extensive comparisons of kinase sequences have defined a common catalytic domain, ranging from 250 to 300 amino acids (3, 4). This domain contains key amino acids that are conserved between kinases and are thought to play an essential role in catalysis.

Recently many protein kinase sequences have been identified either by low-stringency hybridization (5) or by using PCR to amplify sequences between two conserved regions in the kinase domain (6). Using a 500-base-pair (bp) cDNA probe encoding amino acids 193–349 of the $\alpha$ catalytic subunit of the porcine cAMP-PK (7), we isolated several cDNA clones encoding a member of the serine/threonine protein kinase family.‡ Sequence comparisons revealed homology to the cAMP-PK, PKC, and the yeast protein kinase YPK1 (8). We present data on both the expression and activity of this protein kinase.

## MATERIALS AND METHODS

**Molecular Cloning and Sequence Analysis.** Human cDNA libraries derived from MCF-7 cells and WI38 fibroblasts (Clontech and Stratagene) were screened as described (9) using cDNA probe ($\lambda$LLC-PK$_1$rac4B-5) at 1–2 × $10^6$ cpm/ml radiolabeled by the random-priming method (10). Washing was for 2 hr in 0.15 M NaCl/15 mM sodium citrate, pH 7.0 (1× SSC)/0.1% SDS at 60°C. Positive clones were plaque purified, and the *Eco*RI inserts were subcloned for further analysis. cDNA sequences were determined by the dideoxynucleotide chain-termination method (11) by using Sequenase according to the manufacturer's protocols with the universal or reverse-sequencing primers or specific oligonucleotides. All clones were sequenced on both strands with dGTP or with dITP for regions of high G+C content. Sequences were analyzed using the University of Wisconsin Genetics Computer Group software package (12).

**Northern (RNA) Analysis.** Total RNA was isolated as described (13). For Northern analysis, 20 $\mu$g of total RNA was fractionated on a 1% formaldehyde-agarose gel, transferred to Zetaprobe (Bio-Rad) membrane, and hybridized to the 2.3-kilobase (kb) insert from $\lambda$MCFrac8 (specific activity, $\approx 10^9$ cpm/$\mu$g) with 2 × $10^7$ cpm of radiolabeled probe as described (13). After hybridization the blot was washed at 65°C in 1× SSC/0.1% SDS for 2 hr.

***In Vitro* Transcription and Translation.** The *Eco*RI insert from $\lambda$WI38rac71 was subcloned in both orientations into pGEM-4Z (Promega), linearized with *Bam*HI, and used as templates for *in vitro* transcription by SP6 polymerase. The capped RNA ($\approx$1 $\mu$g) was translated *in vitro* by using a rabbit reticulocyte lysate (Stratagene) for 1 hr at 30°C in the presence of 20 $\mu$Ci of [$^{35}$S]methionine (1 Ci = 37 GBq). Samples (2.5 $\mu$l) were analyzed by 12% SDS/PAGE (14) and subjected to autoradiography for 2 hr at −70°C with two intensifying screens.

**Immunization and Purification of rac-Specific Antisera.** Rabbits were immunized by s.c. injection with 1 mg of rac-specific peptide (FPQFSYSASSTA) coupled to keyhole limpet hemocyanin (15). Antisera were purified by precipitation with 50% $(NH_4)_2SO_4$ followed by affinity chromatography on rac-peptide coupled to Affi-Gel 15 (Bio-Rad). The rac-specific antisera were eluted from the column by using

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: cAMP-PK, cAMP-dependent protein kinase; PKC, protein kinase C; MBP, myelin basic protein.

*Present address: Institut fur Immunologie and Virologie der Universität Zürich, Gloriastrasse 30, CH-8028 Zürich, Switzerland.
†To whom reprint requests should be addressed.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M63167).

0.2 M glycine, pH 1.85, and dialyzed against phosphate-buffered saline.

**Immunoanalysis.** Cell extracts were prepared by lysis in 50 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/0.2% Triton X-100. Samples (40 μg of protein) were subjected to 12% SDS/PAGE and electroblotted (16) to Immobilon membranes (Millipore) for 75 min at 300 mA. The membranes were then probed with the rac-specific peptide antisera and visualized using an $^{125}$I-labeled donkey anti-rabbit second antibody (0.1 μCi/ml; Amersham) followed by autoradiography for 2 days at −70°C with two intensifying screens.

**Immunoprecipitation and Assay of rac Protein Kinase.** Cell extracts (≈2 mg/ml), prepared as described above, were precleared by incubation for 1 hr with Pansorbin (Calbiochem) and then incubated with the affinity-purified rac-specific antisera (3 μg) for 2 hr at 0°C. Immunoprecipitates were collected by using protein A-Sepharose (Pharmacia) and extensively washed with lysis buffer. Finally, the immunoprecipitates were washed with 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1 mM dithiothreitol before performing kinase assays. The kinase incubation mixture (50 μl) was 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1 mM dithiothreitol/1 μM protein kinase inhibitor (PKI) peptide (17)/25 μg of histone H1 (Sigma fraction IIIS) or 25 μg of myelin basic protein (MBP, Sigma)/50 μM [γ-$^{32}$P]ATP (800 cpm/pmol)/immunoprecipitate from 50 μl of cell-free extract, corresponding to 100 μg of protein. Other substrates were used as indicated in Table 1. After incubation at 30°C for 30 min samples were analyzed by 12% SDS/PAGE and then autoradiographed. For measurement of protein kinase activity the phosphorylated substrate bands were excised from the dried gel, and radioactivity was counted by scintillation spectrophotometry.

## RESULTS

**Isolation of Clones Encoding rac Kinase.** A 500-bp cDNA probe encoding residues 193–349 of the α catalytic subunit of the porcine cAMP-PK (7) was used at low stringency to screen a cDNA library derived from the porcine kidney cell line LLC-$PK_1$. A partial clone (λLLC-$PK_1$rac4B-5) isolated by this method was sequenced (unpublished data) and shown to encode a serine/threonine protein kinase, termed rac (related to the A and C kinases; see below). The insert from this clone was subsequently used to screen cDNA libraries derived from the human epithelial cell line, MCF-7, and WI38 human lung fibroblasts. Several clones were isolated that encoded the same protein as that identified from LLC-$PK_1$ cells. The human cDNAs used for DNA sequence analysis and further characterization are shown in Fig. 1*A*.

Table 1. Phosphorylation of substrates by rac protein kinase

| Substrate | Concentration, mg/ml | Phosphorylation, % relative to MBP |
|---|---|---|
| MBP | (0.5) | 100 |
| Glycogen synthetase | (0.2) | 100 |
| Regulatory subunit II of cAMP-PK | (0.12) | 82 |
| Histone H1 | (0.5) | 43 |
| 40S ribosomal subunit | (0.5) | 23 |
| Protamine sulphate | (0.5) | 21 |
| Microtubule-associated protein 2 | (0.2) | 10 |
| Histone H2A | (0.5) | 0 |
| Regulatory subunit I of cAMP-PK | (0.12) | 0 |
| Phosphorylase | (0.25) | 0 |

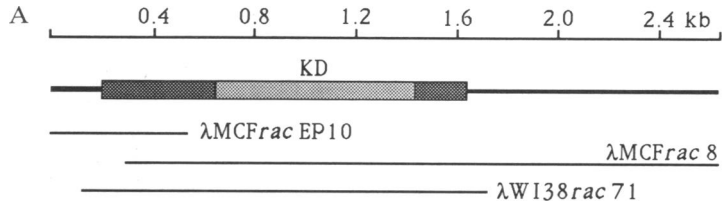
Various substrates were tested in a kinase assay by using rac protein kinase immunoprecipitated from MCF-7 cell extracts with the peptide-specific antisera. Levels of phosphorylation, obtained from three independent immunoprecipitations, are expressed in relation to MBP, which is set at 100%. Numbers in parentheses indicate the concentration of substrate used. Sources of substrates were as follows: MBP, histone H1 (HIIIS), histone H2A (HIIAS), and protamine sulfate were purchased from Sigma; cAMP-PK regulatory subunits I and II and phosphorylase were purified from rabbit skeletal muscle; glycogen synthetase purified from rabbit skeletal muscle was from J. Woodgett (Ludwig Cancer Institute, London); 40S ribosomal subunits purified from rat liver were from G. Thomas (Friedrich Miescher-Institut); microtubule-associated protein 2 purified from rat brain was from A. Matus (Friedrich Miescher-Institut).

**DNA Sequence Analysis of Human rac Protein Kinase.** Complete sequence analysis of the overlapping *Eco*RI fragments of λMCFracEP10 and λMCFrac8 gave a 2.6-kb pair cDNA, with an open reading frame of 480 amino acids from nucleotides 199–1641. Sequence data from λWI38rac71 confirmed that the cDNA encoded the same protein and was a full-length coding clone. The sequence was identical to that obtained from the two overlapping clones but contained only 85 bp of 5′-untranslated region and 127 bp of 3′-untranslated region. The complete coding nucleotide sequence and the predicted amino acid sequence of rac kinase are shown in Fig. 1*B*. The molecular weight of the predicted polypeptide was 55,716. The methionine initiator codon (nucleotides 199–201) was in a favorable position for translation initiation (18). λMCFrac8 lacked a polyadenylylated tail and also lacked a polyadenylylation signal, suggesting that the cDNA did not contain the full 3′-untranslated region.

The predicted protein encoded by the rac kinase cDNA appears to be a member of the serine/threonine protein kinase family, based on the following observations. The sequence motif -Gly-Lys-Gly-Thr-Phe-Gly-, at positions 157–162 with a lysine residue at position 179, fits exactly with the consensus sequence for an ATP-binding site. Other residues thought to be involved in ATP binding are -Asp-Phe-Gly- (19) at positions 292–294, which is the most highly conserved region in all known kinases. Another sequence indicative of a protein kinase catalytic domain is the motif -Ala-Pro-Glu- (4) found at positions 317–319. Also present are two consensus sequences -Gly-Thr-Pro-Glu-Tyr-Leu-Ala-Pro-Glu- (at positions 311–319) and -Asp-Leu-Lys-Leu-Glu-Asn- (at positions 274–279) that distinguish serine/threonine-specific from tyrosine-specific protein kinases (5).

Comparison of the predicted peptide sequence of rac kinase with other protein kinases revealed a considerable degree of similarity to rat PKCε (20), the α catalytic subunit of the bovine cAMP-PK (21), and the yeast protein kinase YPK1, a serine/threonine protein kinase identified from *Saccharomyces cerevisiae* (8). The overall similarities between the kinase domain of rac protein kinase and those of PKCε, α catalytic subunit of cAMP-PK, and YPK1 were 73%, 68%, and 71%, respectively.

**Identification of an Initiator Methionine Residue.** To confirm the predicted open reading frame, rac protein kinase transcripts were synthesized *in vitro* and translated in a cell-free system in the presence of [$^{35}$S]methionine. The sense transcript produced a major polypeptide with an estimated $M_r$ of 58,000 (Fig. 2*A*). As this $M_r$ correlated well with that predicted from the nucleotide sequence, the methionine residue at nucleotides 199–201 was presumed to be the correct initiator methionine. The presence of minor low-molecular weight polypeptides (≈20% of incorporated label) suggests initiation from internal methionines (22). No polypeptides of comparable size were synthesized from the antisense transcript or in the absence of exogenous RNA (Fig. 2*A*). The *in vitro*-translated products were recognized and immunoprecipitated by antibodies raised against a carboxyl-terminal peptide (residues 469–480) (Fig. 2*B*).




B

```
1    ATCCTGGGACAGGGCACAGGGCCATCTGTCACCAGGGGCTTAGGGAAGGCCGAGCCAGCCTGGGTCAAAGAAGTCAAAGGGGCTGCCTGG   90
91   AGGAGGCAGCCTGTCAGCTGGTGCATCAGAGGCTGTGGCCAGGCCAGCTGGGCTCGGGGAGCGCCAGCCTGAGAGGAGCGCGTGAGCGTC  180
                    1                   10                  20
181  GCGGGAGCCTCGGGCACCATGAGCGACGTGGCTATTGTGAAGGAGGGTTGGCTGCACAAACGAGGGGAGTACATCAAGACCTGGCGGCCA  270
                    MetSerAspValAlaIleValLysGluGlyTrpLeuHisLysArgGlyGluTyrIleLysThrTrpArgPro
                30                  40                  50
271  CGCTACTTCCTCCTCAAGAATGATGGCACCTTCATTGGCTACAAGGAGCGGCCGCAGGATGTGGACCAACGTGAGGCTCCCCTCAACAAC  360
     ArgTyrPheLeuLeuLysAsnAspGlyThrPheIleGlyTyrLysGluArgProGlnAspValAspGlnArgGluAlaProLeuAsnAsn
            60                  70                  80
361  TTCTCTGTGGCGCAGTGCCAGCTGATGAAGACGGAGCGGCCCCGGCCCAACACCTTCATCATCCGCTGCCTGCAGTGGACCACTGTCATC  450
     PheSerValAlaGlnCysGlnLeuMetLysThrGluArgProArgProAsnThrPheIleIleArgCysLeuGlnTrpThrThrValIle
        90                  100                 110
451  GAACGCACCTTCCATGTGGAGACTCCTGAGGAGCGGGAGGAGTGGACAACCGCCATCCAGACTGTGGCTGACGGCCTCAAGAAGCAGGAG  540
     GluArgThrPheHisValGluThrProGluGluArgGluGluTrpThrThrAlaIleGlnThrValAlaAspGlyLeuLysLysGlnGlu
    120                 130                 140
541  GAGGAGGAGATGGACTTCCGGTCGGGCTCACCCAGTGACAACTCAGGGGCTGAAGAGATGGAGGTGTCCCTGGCCAAGCCCAAGCACCGC  630
     GluGluGluMetAspPheArgSerGlySerProSerAspAsnSerGlyAlaGluGluMetGluValSerLeuAlaLysProLysHisArg
150                 160                 170
631  GTGACCATGAACGAGTTTGAGTACCTGAAGCTGCTGGGCAAGGGCACTTTCGGCAAGGTGATCCTGGTGAAGGAGAAGGCCACAGGCCGC  720
     ValThrMetAsnGluPheGluTyrLeuLysLeuLeuGlyLysGlyThrPheGlyLysValIleLeuValLysGluLysAlaThrGlyArg
180                 190                 200
721  TACTACGCCATGAAGATCCTCAAGAAGGAAGTCATCGTGGCCAAGGACGAGGTGGCCCACACACTCACCGAGAACCGCGTCCTGCAGAAC  810
     TyrTyrAlaMetLysIleLeuLysLysGluValIleValAlaLysAspGluValAlaHisThrLeuThrGluAsnArgValLeuGlnAsn
210                 220                 230
811  TCCAGGCACCCCTTCCTCACAGCCCTGAAGTACTCTTTCCAGACCCACGACCGCCTCTGCTTTGTCATGGAGTACGCCAACGGGGGCGAG  900
     SerArgHisProPheLeuThrAlaLeuLysTyrSerPheGlnThrHisAspArgLeuCysPheValMetGluTyrAlaAsnGlyGlyGlu
240                 250                 260
901  CTGTTCTTCCACCTGTCCCGGGAACGTGTGTTCTCCGAGGACCGGGCCCGCTTCTATGGCGCTGAGATTGTGTCAGCCCTGGACTACCTG  990
     LeuPhePheHisLeuSerArgGluArgValPheSerGluAspArgAlaArgPheTyrGlyAlaGluIleValSerAlaLeuAspTyrLeu
270                 280                 290
991  CACTCGGAGAAGAACGTGGTGTACCGGGACCTCAAGCTGGAGAACCTCATGCTGGACAAGGACGGGCACATTAAGATCACAGACTTCGGG 1080
     HisSerGluLysAsnValValTyrArgAspLeuLysLeuGluAsnLeuMetLeuAspLysAspGlyHisIleLysIleThrAspPheGly
300                 310                 320
1081 CTGTGCAAGGAGGGGATCAAGGACGGTGCCACCATGAAGACCTTTTGCGGCACACCTGAGTACCTGGCCCCCGAGGTGCTGGAGGACAAT 1170
     LeuCysLysGluGlyIleLysAspGlyAlaThrMetLysThrPheCysGlyThrProGluTyrLeuAlaProGluValLeuGluAspAsn
330                 340                 350
1171 GACTACGGCCGTGCAGTGGACTGGTGGGGGCTGGGCGTGGTCATGTACGAGATGATGTGCGGTCGCCTGCCCTTCTACAACCAGGACCAT 1260
     AspTyrGlyArgAlaValAspTrpTrpGlyLeuGlyValValMetTyrGluMetMetCysGlyArgLeuProPheTyrAsnGlnAspHis
360                 370                 380
1261 GAGAAGCTTTTTGAGCTCATCCTCATGGAGGAGATCCGCTTCCCGCGCACGCTTGGTCCCGAGGCCAAGTCCTTGCTTTCAGGGCTGCTC 1350
     GluLysLeuPheGluLeuIleLeuMetGluGluIleArgPheProArgThrLeuGlyProGluAlaLysSerLeuLeuSerGlyLeuLeu
390                 400                 410
1351 AAGAAGGACCCCAAGCAGAGGCTTGGCGGGGGCTCCGAGGACGCCAAGGAGATCATGCAGCATCGCTTCTTTGCCGGTATCGTGTGGCAG 1440
     LysLysAspProLysGlnArgLeuGlyGlyGlySerGluAspAlaLysGluIleMetGlnHisArgPhePheAlaGlyIleValTrpGln
420                 430                 440
1441 CACGTGTACGAGAAGAAGCTCAGCCCACCCTTCAAGCCCCAGGTCACGTCGGAGACTGACACCAGGTATTTTGATGAGGAGTTCACGGCC 1530
     HisValTyrGluLysLysLeuSerProProPheLysProGlnValThrSerGluThrAspThrArgTyrPheAspGluGluPheThrAla
450                 460                 470
1531 CAGATGATCACCATCACACCACCTGACCAAGATGACAGCATGGAGTGTGTGGACAGCGAGCGCAGGCCCCACTTCCCCCAGTTCTCCTAC 1620
     GlnMetIleThrIleThrProProAspGlnAspAspSerMetGluCysValAspSerGluArgArgProHisPheProGlnPheSerTyr
480
1621 TCGGCCAGCAGCACGGCCTGAGGCGGCGGTGGACTGCGCTGGACGATAGCTTGGAGGGATGGAGAGGCGGCCTCGTGCCATGATCTGTAT 1710
     SerAlaSerSerThrAla
1711 TTAATGGTTTTTATTTCTCGGGTGCATTTGAGAGAAGCCACGCTGTCCTCTCGAGCCCAGATGGAAAGACGTTTTTGTGCTGTGGGCAGC 1800
1801 ACCCTCCCCCGCAGCGGGGTAGGGAAGAAAACTATCCTGCGGGTTTTAATTTATTTCATCCAGTTTGTTCTCCGGGTGTGGCCTCAGCCC 1890
1891 TCAGAACAATCCGATTCACGTAGGGAAATGTTAAGGACTTCTACAGCTATGCGCAATGTGGCATTGGGGGGCCGGGCAGGTCCTGCCCAT 1980
1981 GTGTCCCCTCACTCTGTCAGCCAGCCGCCCTGGGCTGTCTGTCACCAGCTATCTGTCATCTCTCTGGGGCCCTGGGCCTCAGTTCAACCT 2070
2071 GGTGGCACCAGATGCAACCTCACTATGGTATGCTGGCCAGCACCCTCTCCTGGGGGTGGCAGGCACACAGCAGCCCCCCAGCACTAAGGC 2160
2161 CGTGTCTCTGAGGACGTCATCGGAGGCTGGGCCCCTGGGATGGGACCAGGGATGGGGGATGGGCCAGGGTTTACCCAGTGGGACAGAGGA 2250
2251 GCAAGGTTTAAATTTGTTATTGTGTATTATGTTGTTCAAATGCATTTTGGGGGTTTTTAATCTTTGTGACAGGAAAGCCCTCCCCCTTCC 2340
2341 CCTTCTGTGTCACAGTTCTTGGTGACTGTCCCACCGGAGCCTCCCCCTCAGATGATCTCTCCACGGTAGCACTTGACCTTTTCGACGCTT 2430
2431 AACCTTTCCGCTGTCGCCCCAGGCCCTCCCTGACTCCCTGTGGGGGTGGCCATCCCTGGGCCCCTCCACGCCTCCTGGCCAGACGCTGCC 2520
2521 GCTGCCGCTGCACCACGGCGTTTTTTTACAACATTCAACTTTAGTATTTTTACTATTATAATATAATATGGAACCTTCCCTCCAAATTCT 2610
```

FIG. 1. (*A*) cDNAs encoding human rac protein kinase isolated from MCF-7 and WI38 libraries. Schematic representation of the rac kinase mRNA with the coding sequence (shaded box) flanked by 5′- and 3′-untranslated regions (solid lines) is shown, where KD represents the kinase domain. cDNAs used for sequence analysis of rac protein kinase are shown below. (*B*) Nucleotide sequence and predicted amino acid sequence of human rac protein kinase. The predicted amino acid sequence (numbered 1–480) is shown above the respective codon in the nucleotide sequence (numbered 1–2610). Arrows over the nucleotide sequence (positions 132 and 1760) denote ends of clone λWI38rac71.

**Northern Analysis of rac Protein Kinase Expression in Human Cell Lines.** The pattern of expression of rac protein kinase was analyzed in several human cell lines. A single transcript of 3.2 kb was expressed in all cell types analyzed (Fig. 3), although the level of expression varied greatly. MCF-7 cells had the most abundant rac kinase transcript—≈300 times more mRNA than found in A1146 cells, which had the lowest level of expression.

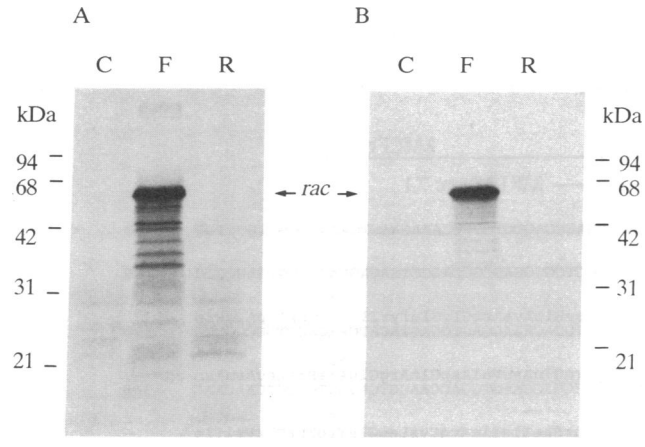



FIG. 2. *In vitro* translation of human rac protein kinase and immunoprecipitation of the rac protein kinase by a peptide-specific antisera. (*A*) SDS/PAGE analysis of $^{35}$S-labeled protein after translation of rac kinase mRNA, as described. Lanes: C, no exogenous RNA; F, sense transcript; R, antisense transcript. Molecular size markers in kDa are shown. (*B*) Immunoprecipitation of rac kinase mRNA translation products. Samples were immunoprecipitated by using the rac-specific peptide antisera and analyzed by 12% SDS/PAGE followed by fluorography. Loading order is as in *A*.

**Immunoanalysis of the rac Protein Kinase.** The level of expression of the rac protein kinase in MCF-7, HeLa, and A431 cells was compared by immunoblotting with an affinity-purified peptide antisera. Fig. 4*A* shows that each cell type expressed a protein of $M_r$ 59,000 that was recognized by the rac-specific peptide antisera. The level of expression was ≈ 4-fold higher in MCF-7 cells than in HeLa or A431 cells, which had approximately equal protein levels. The difference in level of expression at the protein level was similar to that at the RNA level, where an 8-fold difference was seen between MCF-7 and HeLa cells.

**Identification of rac Protein Kinase Activity.** Due to the high level of mRNA and protein in MCF-7 cells we used this cell line to identify the rac protein kinase activity with affinity-purified rac-specific antisera. The kinase activity immunoprecipitated from MCF-7 cell-free extracts was shown to phosphorylate histone H1 and MBP (Fig. 4 *B* and *C*). The level of phosphorylation depended on the amount of antisera used during immunoprecipitation (Fig. 4*C*, lanes 1–6). Furthermore, addition of the immunogenic peptide to 2 mM during immunoprecipitation reduced the level of MBP phosphorylation by 97% (Fig. 4*C*, lane 7), whereas addition of another peptide taken from the predicted rac kinase sequence (RLPFYNQDHEKL) to 2 mM had no effect (Fig. 4*C*, lane 8). Immunoprecipitation of this kinase activity could also be inhibited by the addition of bacterially expressed recombinant protein (unpublished data). From these observations we assume that this assay was specific for the rac protein kinase.

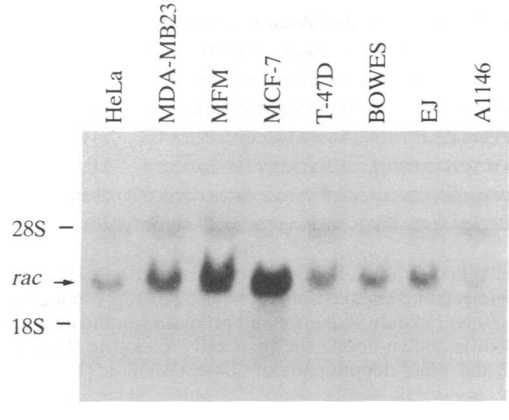



FIG. 3. Northern blot analysis of rac protein kinase transcripts in human cell lines as described in text. Positions of the 28S and 18S rRNA bands are shown at left; position of the rac kinase transcript is marked by an arrow.

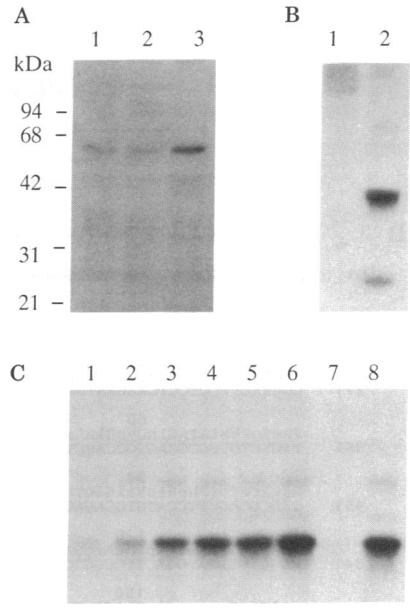



FIG. 4. Identification of rac protein and kinase activity in human cells. (*A*) Western (immuno)blot analysis of rac protein expression in A431, HeLa, and MCF-7 cells. Cell extracts were subjected to SDS/PAGE, blotted, and probed with the rac-specific antisera, as described. Molecular size markers in kDa are shown at left. Lanes: 1, A431; 2, HeLa; 3, MCF-7. (*B*) Identification of rac protein kinase activity by using histone H1 as substrate. Lanes: 1, no exogenous substrate; 2, 25 μg of histone H1. (*C*) Specificity of rac protein kinase assay. Increased amounts of antibody were added to MCF-7 cell extracts during immunoprecipitation. Kinase activity was assayed by using MBP as substrate (see text). Lanes: 1–6, 0.5 μg, 1.0 μg, 1.5 μg, 2.0 μg, 2.5 μg, and 3 μg of affinity-purified antisera, respectively; 7, 3 μg of antisera plus 2 mM immunogenic rac-specific peptide; 8, 3 μg of antisera plus 2 mM nonimmunogenic peptide.

Quantitation of rac protein kinase activity, either by counting radioactivity in the phosphorylated bands or by filter assay, showed that the specific activity in cell-free extracts was 2.5 pmol/mg per min for histone H1 and 6 pmol/mg per min for MBP. Addition of 5 μM cAMP or 2 mM EGTA or the pseudosubstrate synthetic inhibitor of PKC (23) showed no effect on activity. Similarly, addition of protein kinase inhibitor peptide (routinely used in all assays) had no effect. Therefore, the observed kinase activity appears distinct from either the cAMP-PK or PKC.

Physiological substrates were used to determine which proteins may be targets for the rac protein kinase *in vivo*. Phosphorylation values for each substrate, shown in Table 1, are expressed in relation to MBP, which was arbitrarily set at 100%. The highest rates of phosphorylation were seen by using MBP, glycogen synthetase, and the cAMP-PK regulatory subunit II. Lower rates were seen with histone H1, 40S ribosomal subunit, protamine, and microtubule-associated protein 2, whereas no phosphorylation was detected with histone H2A, cAMP-PK regulatory subunit I, or phosphorylase as substrates.

## DISCUSSION

In this paper we report the identification of another member of the serine/threonine protein kinase of the second-messenger subfamily. The predicted amino acid sequence of

this protein kinase, termed rac, shows a similar degree of homology to both the cAMP-PK and PKC. The rac protein kinase does not appear to be another isoform of the catalytic subunit of cAMP-PK for the following reasons. (*i*) The catalytic subunit isotypes of human cAMP-PK (24) show 90–96% homologies to each other, whereas the predicted rac kinase product shows only 67–68% homology to these subunits in the kinase domain. (*ii*) The catalytic subunits of the cAMP-PK consist of 351 amino acids containing 42 residues upstream of the kinase domain, whereas rac protein kinase has a similar-sized kinase domain but 148 residues in the amino-terminal domain.

PKC and the cGMP-dependent protein kinase (cGMP-PK) (25) both contain a kinase domain at the carboxyl terminal of the protein and an apparent regulatory domain, ranging from 300 to 630 amino acids in length, at the amino terminal. It is possible that the amino-terminal 148 amino acids of rac protein kinase form an analogous regulatory domain. However, members of the PKC family have homologous regions within the regulatory domain containing at least one cysteine-rich sequence (20). rac protein kinase shows no homology in this region to either PKC or the cGMP-PK. Alternatively, it may be that these 148 amino acids are not sufficient to form a similar regulatory domain and that rac protein kinase, like the cAMP-PK, has separate catalytic and regulatory subunits.

The rac protein kinase transcript is expressed in all cell lines so far analyzed. It has also been identified in porcine brain, heart, liver, kidney, muscle, and ovary (unpublished data). One striking feature of the pattern of expression is the 300-fold difference seen between the levels of expression in MCF-7 cells and A1146 cells. This expression pattern may be due to either high transcription rate or gene amplification.

The assay used to determine the kinase activity appears specific for the rac protein because it is antibody dependent and is inhibited by the peptide used for immunization but not by another peptide from the rac protein sequence. The activity associated with rac protein kinase under these conditions was estimated at 2.5–6 pmol/mg per min by using histone H1 and MBP, respectively. The specific activities obtained for other protein kinases in cell-free extracts—e.g., 2–4 nmol/mg per min for the cAMP-PK, 3.5 nmol/mg per min for PKC (26), and 87 pmol/mg per min for S6 kinase (27)—suggest that rac protein kinase activity is low in our assay system. It should be noted that all these specific activities have been estimated in different cell systems and, therefore, may not be directly comparable. It is, therefore, possible that rac protein kinase has been overlooked due to its apparent low activity.

Several explanations exist to account for the apparently low activity of the rac protein kinase. (*i*) The presence of the antibodies in the immunoprecipitate may inhibit the rac kinase activity. (*ii*) Immunoprecipitation may dissociate the rac protein kinase from an activator protein, such as seen for cyclin-mediated activation of the cdc2 kinase (28). (*iii*) The rac protein kinase activity may be activated by phosphorylation. Several protein kinases are up-regulated by phosphorylation, such as mitogen-stimulated S6 kinase (29) and microtubule-associated protein 2 kinase (30).

Characterization of *in vivo* substrates for rac protein kinase may shed light onto its possible cellular functions. The data in Table 1 show that rac protein kinase can phosphorylate a number of physiological substrates and also that this protein kinase activity is distinct from either cAMP-PK or PKC. These two protein kinases are known to be involved in separate signal-transduction pathways and respond to different second messengers. A critical step in the establishment of the physiological role of rac protein kinase will be identification of a second messenger that modulates its activity and, hence, identification of the signal-transduction pathway involved.

We thank R. Adavani and D. Suput for preliminary sequence analysis of the original cDNA, F. Fischer and W. Zurcher for synthesis of the oligonucleotides and peptides, Prof. P. Chambon (Institut National de la Sarté et de la Recherche Médicale, Strasbourg) for the MCF-7 cDNA library, and W. Filipowicz, K. Ballmer-Hofer, and Y. Nagamine for critical comments on the manuscript.

1. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) *Annu. Rev. Biochem.* **56,** 567–613.
2. Yarden, Y. & Ullrich, A. (1988) *Annu. Rev. Biochem.* **57,** 443–477.
3. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241,** 42–52.
4. Hunter, T. & Cooper, J. A. (1986) in *The Enzymes,* eds. Boyer, P. D. & Krebs, E. G. (Academic, Orlando, FL), 3rd Ed., Vol. 17, pp. 191–246.
5. Hanks, S. K. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 388–392.
6. Wilks, A. F. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1603–1607.
7. Adavani, S. R., Schwarz, M., Showers, M. O., Maurer, R. A. & Hemmings, B. A. (1987) *Eur. J. Biochem.* **167,** 221–226.
8. Maurer, R. A. (1988) *DNA* **7,** 469–474.
9. Stone, S. R., Hofsteenge, J. & Hemmings, B. A. (1987) *Biochemistry* **26,** 7215–7220.
10. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
11. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
12. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395.
13. Khew-Goodall, Y. & Hemmings, B. A. (1988) *FEBS Lett.* **238,** 265–268.
14. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
15. Doolittle, R. F. (1986) *Of URFs and ORFs: A Primer on How to Analyze Derived Amino Acid Sequences* (University Science Books, Mill Valley, CA).
16. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
17. Scott, J. D., Fischer, E. H., Demaille, J. G. & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4379–4383.
18. Kozak, M. (1984) *Nucleic Acids Res.* **12,** 857–872.
19. Brenner, S. (1987) *Nature (London)* **329,** 21.
20. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. (1988) *J. Biol. Chem.* **263,** 6927–6932.
21. Shoji, S., Parmelee, D. C., Wade, R. D., Kumar, S., Ericsson, L. H., Walsh, K. A., Neurath, H., Long, G. L., Demailee, J. G., Fischer, E. H. & Titani, K. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 848–851.
22. Benovic, J. L., DeBlasi, A., Stone, W. C., Caron, M. G. & Lefkowitz, R. J. (1989) *Science* **246,** 235–240.
23. House, C. & Kemp, B. E. (1987) *Science* **238,** 1726–1728.
24. Beebe, S. J., Øyen, O., Sandberg, M., Frøysa, A., Hansson, V. & Jahnsen, T. (1990) *Mol. Endocrinol.* **4,** 465–475.
25. Takio, K., Wade, R. D., Smith, S. B., Krebs, S. G., Walsh, K. A. & Titani, K. (1984) *Biochemistry* **23,** 4207–4218.
26. Parker, P. J., Stabel, S. & Waterfield, M. D. (1984) *EMBO J.* **3,** 953–959.
27. Jenö, P., Jäggi, N., Luther, H., Siegmann, M. & Thomas, G. (1989) *J. Biol. Chem.* **264,** 1293–1297.
28. Draetta, G., Luca, F., Westerndorf, J., Brizuela, L., Ruderman, J. & Beach, D. (1989) *Cell* **56,** 829–838.
29. Ballou, L. M., Siegmann, M. & Thomas, G. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7154–7158.
30. Ray, L. B. & Sturgill, T. W. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3753–3757.