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Molecular cloning and sequencing of rat Cdc42 GTPase cDNA

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Abstract

Cdc42 is a member of the Rho family of small GTPase and plays an important role in intracellular signaling pathways regulating cell morphology, motility and stimulation of DNA synthesis. We have isolated cDNA encoding Cdc42 from a rat brain cDNA library using PCR-cloning strategy. The sequence of isolated gene revealed an open reading frame of 576 nucleotides with the potential to encode a protein of 191 amino acids with a predicted molecular weight of 21 kD. The resulting sequence was incorporated into the GenBank with accession number, AF205635. Sequence analysis revealed that overall cDNA sequence identity is 96% with human G25K and 52% with rat Chp, a homologue of the GTPase human Cdc42Hs, and having one nucleotide difference from the mouse Cdc42. However, putative protein sequence was identical to the mouse and human brain Cdc42Hs. On expression of the cDNA in COS-7 cells, a protein molecular weight of 21 kD was detected in immunoblotting using anti-human Cdc42 antibodies. Therefore, these results suggest that the cDNA we are reporting is most likely the rat homologue of the GTPase human Cdc42.

Keywords: GTPase, Cdc42, PCR Cloning, COS-7 cell, Rho

Introduction

Small GTPases are essential components in a number of biological processes, including the hormonal regulation of adenylate cyclase and phospholipase, visual transduction, protein trafficking, secretion and cell growth (Shinjo *et al*., 1990; Johnson, 1999). Cdc42 is a member of the Rho family of small GTPase and plays an important role in intracellular signaling pathways that influence cell morphology, motility and stimulation of DNA synthesis (Nobes and Hall, 1995; Olson *et al*., 1995). Like other GTPases, Cdc42 can exist in an active GTP-bound state and an inactive GDP-bound state (Bourne *et al*., 1990). In yeast, Cdc42 acts as a regulator of the cell division cycle (Adams *et al*., 1990; Oehlen and Cross, 1998). The mammalian homologue of yeast Cdc42 has been shown to regulate the formation of the actin structure filopodia in Swiss 3T3 cells (Kozma *et al*., 1995) and cell polarity-dependent processes in T cell activation (Stowers *et al*., 1995). Human Cdc42 was initially isolated in platelets and placenta, and serves as a substrate for phosphorylation by epidermal growth factor receptor (Evans *et al*., 1986). In addition, it has recently been shown that Cdc42 may act downstream of Arf in some types of intracellular trafficking events (Radhakrishna *et al*., 1999) and can act synergistically with Arf to stimulate phospholipase D activity in mammalian cells (Han *et al*., 1996). Structural and functional Cdc42 homologues have subsequently been characterized in the pathogenic *yeast Candida albicans* (Mirbod *et al*., 1997), *S. pombe* (Miller and Johnson, 1994), *C. elegans* (Chen *et al*., 1993), *Drosophila* (Luo *et al*., 1994), chicken (*Gallus gallus*) cochlea (Gong *et al*., 1997), mouse (*Mus musculus*) liver (Gong *et al*., 1997) and brain (Marks and Kwiatkowski, 1996). These homologues are 80 to 95% identical in predicted amino acid sequence (Johnson, 1999) and it suggests that Cdc42 may have conserved functions in various species. In the rat, although the Chp, a homologue of the GTPase human Cdc42Hs, was cloned, its overall sequence identity is 52% with Cdc42Hs (Aronheim *et al*., 1998). We have recently purified Cdc42 from rat brain cytosol (Han *et al*., 1998). Here we report the cloning and sequencing of cDNA which encodes the rat (*Rattus norverticus*) Cdc42 from a rat brain cDNA library. Using the PCR-cloning technique, we successfully cloned cDNA for rat Cdc42. The cDNA obtained could be expressed in COS-7 cells, and Western blot analysis showed that the protein was highly reactive to anti-human Cdc42 antibodies.

Materials and Methods

PCR cloning of the rat Cdc42 cDNA

A rat brain cDNA library in pB42AD was used as the

template for PCR-cloning of rat Cdc42 cDNA. Oligonucleotide primers were arranged as follows based on the mouse Cdc42 cDNA: 5'-CCGGGATCCATGCAGACAA-TTAAGTGTGTTGTT-3' (sense) and 5'-GCCGAATTC-TTAGAATATACAGCACTTCCTTTT-3' (antisense) corresponding to nucleotides 1-576 of the full length of mouse Cdc42. The sequences on underlines are *Bam*H I (GGATCC) and *Eco*R I (GAATTC) recognition sites which were added to the insert PCR products into expression vector, pcDNA3.1 (Invitrogen). The thermal cycler program used for PCR amplification consisted of an initial denaturation step at 94°C followed by 30 cycles consisting of 1 min denaturation at 94°C, 1 min annealing at 54°C and a 45 sec elongation at 72°C. A final 10 min elongation at 72°C followed. The PCR products were separated on 1% agarose gels and recovered from gels using JETsorb (Genomed). For the sequence analysis, the PCR product was cloned into TA-pGEM cloning vector (Promega), and *E. coli* bacterial strain JM109 was used as a host.

DNA sequencing

Plasmid DNA was isolated from recombinant colonies with the plasmid mini-prep kit (Promega). The plasmid DNA was purified and sequenced by dideoxy chain termination method using T7/SP6 sequencing primers and BigDye Terminator Cycle Sequencing Kit with ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). The sequences were subsequently analyzed with the program DNASIS version 2.1 (Hitachi Software).

Cell culture

COS-7 cell was obtained from American Type Culture Collection (ATCC) and grown in DMEM (Gibco) supplement with 10% heat-inactivated fetal bovine serum, penicillin (100 unit/ml) and streptomycin (100 μg/ml) at 37°C in CO_2 incubators.

Transient transfection of COS-7 Cells using LipofecAMINE

The cDNA fragments encoding Cdc42 were subcloned in the mammalian expression vector, pcDNA3.1. The TA-pGEM cloning vector containing Cdc42 cDNA were double-digested with *Bam*H I and *Not* I, and the resulting cDNA were ligated into the corresponding sites in pcDNA3.1 using T_4 DNA ligase. COS-7 cells were transfected with the recombinant plasmid pcDNA3.1-Cdc42 using LipofectAMINE reagent (Gibco BRL) according to the manufacturer's protocol. Briefly, cells were grown to 80% confluence in 100-mm dishes. cDNA (2 μg) and LipofecAMINE (20 μl) were separately diluted in serum-free DMEM to 200 μl volumes, mixed, and incubated at room temperature for 1 h. Cells were washed twice with PBS and overlaid with 5 ml of serum-free DMEM to which the DNA-lipid complexes were added. After 6 h, the DNA-lipid complex was replaced with a growth medium containing 10% FBS and 500 μg/ml geneticin, and the cells were incubated for 72 h.

Immunoblot analysis

Cells were washed twice with phosphate-buffered saline

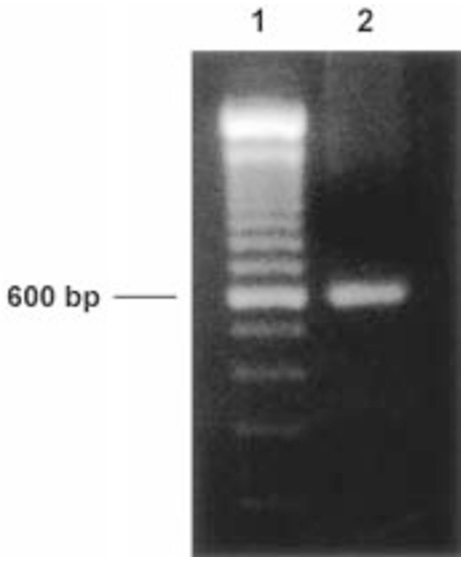

Figure 1. Analysis of amplified PCR products by 1.5% agarose gel electrophoresis. Lane 1: 100 bp size marker, Lane 2: PCR product from rat brain library obtained after 30 cycles at 94°C 1 min, 54°C 1 min, 72°C 45 sec.

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   1 ATGCAGACAATTAAGTGTGTTGTTGTTGGTGATGGTGCTGTTGGTAAAACATGTCTCCTG
   1  M  Q  T  I  K  C  V  V  V  G  D  G  A  V  G  K  T  C  L  L

  61 ATATCCTACACAACAAACAAATTCCCATCGGAATATGTACCAACTGTTTTTGACAACTAT
  21  I  S  Y  T  T  N  K  F  P  S  E  Y  V  P  T  V  F  D  N  Y

 121 GCAGTCACAGTTATGATTGGTGGAGAGCCATACACTCTTGGACTTTTTGATACTGCAGGG
  41  A  V  T  V  M  I  G  G  E  P  Y  T  L  G  L  F  D  T  A  G

 181 CAAGAGGATTATGACAGACTACGACCGCTAAGTTATCCACAGACAGATGTTTTTCTAGTA
  61  Q  E  D  Y  D  R  L  R  P  L  S  Y  P  Q  T  D  V  F  L  V

 241 TGTTTCTCAGTGGTCTCTCCATCCTCATTTGAAAATGTGAAAGAAAAGTGGGTGCCTGAG
  81  C  F  S  V  V  S  P  S  S  F  E  N  V  K  E  K  W  V  P  E

 301 ATAACTCACCACTGTCCAAAGACTCCTTTCTTGCTTGTTGGGACCCAAATTGATCTCAGA
 101  I  T  H  H  C  P  K  T  P  F  L  L  V  G  T  Q  I  D  L  R

 361 GATGACCCCTCTACTATTGAGAAACTTGCCAAGAACAAACAGAAGCCTATTACTCCAGAG
 121  D  D  P  S  T  I  E  K  L  A  K  N  K  Q  K  P  I  T  P  E

 421 ACTGCTGAAAAGCTGGCGCGGGATCTGAAGGCTGTCAAGTATGTGGAGTGCTCCGCCCTC
 141  T  A  E  K  L  A  R  D  L  K  A  V  K  Y  V  E  C  S  A  L

 481 ACACAGAGAGGTCTGAAGAATGTGTTTGATGAGGCTATCCTAGCTGCCCTCGAGCCTCCG
 161  T  Q  R  G  L  K  N  V  F  D  E  A  I  L  A  A  L  E  P  P

 541 GAAACTCAACCCAAAAGGAAGTGCTGTATATTCTAA
 181  E  T  Q  P  K  R  K  C  C  I  F  *
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Figure 2. Nucleotide and deduced amino acid sequences of the rat brain Cdc42. Nucleotide and amino acid positions are indicated at the left and the termination codon is marked by an *asterisk*. The primers used for PCR span the entire coding region of the cDNA and are *underlined*. The accession number of this cDNA in the GenBank is AF205635.

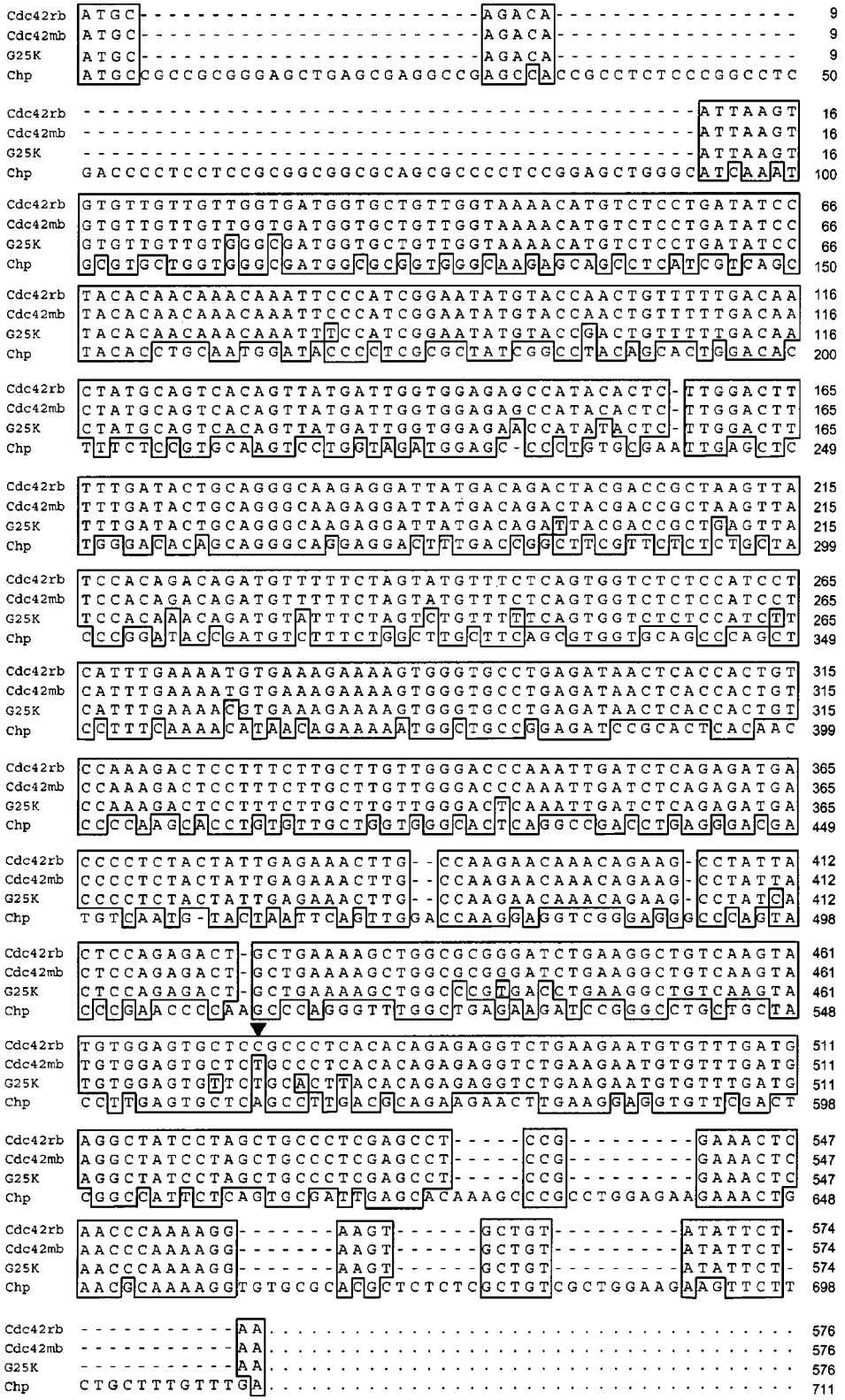

Figure 3. Multiple sequence alignment of Cdc42 or its homologues. The nucleotide sequences of rat brain Cdc42 (Cdc42rb), mouse brain Cdc42 (Cdc42mb), human G25K (G25K, from human fetal brain) and rat Chp (Chp), a homologue of the GTPase human Cdc42Hs were aligned using the program DNASIS Version 2.1. The boxed region denotes the nucleotides identical to those of the rat Cdc42 cDNA and dashes indicate gaps introduced into the sequence to improve alignment.

(PBS) and harvested in 0.5 ml of lysis buffer (50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 mM HEPES, pH 7.5) and disrupted by sonication. Total protein concentration was determined by the Bradford procedure using BSA as a standard. Sample lysate (40 μg) was mixed with sample buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol in 0.125 M Tris, pH 6.8) containing bromophenol blue, boiled for 5 min, and then run on 12% SDS-PAGE. The separated protein was electrophoretically transferred to the nitrocellulose membrane (BA83, Schleicher & Schuell). The membrane was then blocked with 5% BSA in TTBS (Tris-buffered saline containing 0.01% Tween-20) for 1 h and then incubated for another 1 h with mouse anti-human Cdc42 monoclonal antibody at 1 : 1000 dilution. Unbound primary antibodies were removed by three washings with TTBS, and the membrane was incubated with peroxidase-conjugated anti-mouse IgG antibody (Sigma) for 1 h. After washing, the membrane was developed by enhanced chemiluminesence (Amersham Corp.) and exposed for 1 min to Kodak X-AR5 film prior to developing the immunoblots.

Results and Discussion

In this report, we have successfully isolated and sequenced the cDNA for a low molecular weight GTP-binding protein, Cdc42 from a rat brain library. Recently, a homologue of the GTPase human Cdc42Hs, designated Chp, was cloned from a rat pituitary cDNA library (Aronheim *et al*., 1998). However, Chp protein exhibits an overall sequence identity of 52-57% to Cdc42 of other species. To clone the Cdc42 in the rat, we screened an adult rat brain cDNA library using the PCR-cloning technique. Recent studies showed that the TA cloning system is the most efficient cloning system for PCR-amplified DNA fragments with minimal manipulation and ease of handling. Simultaneously, the cloned DNA obtained from the TA cloning system can be directly sequenced without further manipulation (Kern *et al*., 1992; Sarkar *et al*., 1993).

By using the primer based on the mouse Cdc42 cDNA, we obtained a PCR product which was about 600 bp (Figure 1). The resulting PCR product was ligated into a TA-pGEM cloning vector, and the positive clones from PCR reactions were subjected to nucleotide sequencing. Sequencing of the inserted PCR product revealed that it has an open reading frame of 576 bp that codes for 191 amino acids, yielding a predicted M_r 21,000. The nucleotide sequence and the deduced amino acids are shown in Figure 2. Overall cDNA sequence identity is 96% with human G25K, and 52% with rat

Figure 4. Reactivity of the COS-7-expressed rat Cdc42 protein with anti-human Cdc42 antibodies. The rat brain Cdc42 protein was expressed in COS-7 cells with pcDNA 3.1 vector, and cell lysates were subjected to SDS-PAGE. The gel was immnoblotted with anti-human Cdc42 antibodies and the blot was developed with enhanced chemiluminescence. Lane 1: COS-7 cells transfected with vector lacking the cloned cDNA inserts, Lane 2: COS-7 cell expressing cloned rat Cdc42 (100 μg).

Chp, a homologue of the GTPase Cdc42Hs. Especially, comparison of the nucleotide sequences of cloned cDNA to those of mouse brain Cdc42 showed that they were identical except for just one nucleotide difference at position 474 (T → C) (Figure 3). However, putative protein sequence was identical to the mouse and human brain Cdc42 reported by Marks *et al*. and Barfod *et al*., respectively. (GenBank accession No. L78075; M35543). Previous studies have also shown that the Cdc42 family of proteins has 11 members ranging in size from 190 to 192 amino acids. Within this family, there is a very high degree of sequence conservation, ranging from 75% amino acid identity between C. albicans Cdc42p and the human brain isoform G25K to 100% identity between the mouse brain and human brain (G25K) isoforms (Barfod *et al*., 1993; Johnson, 1999).

To confirm our results, we transfected COS-7 cells with the cloned cDNA and investigated for cross-reactivity using anti-human Cdc42 antibody. Successful expression of the rat Cdc42 was achieved using mammalian COS-7 cells. As shown in Figure 4, COS-7 cells transfected with pcDNA3.1-Cdc42 highly expressed the protein, which was able to react strongly with anti-human Cdc42 antibodies. This antibody is reactive with epitope corresponding to amino acids 166-182 mapping near the carboxy terminus of human Cdc42 (identical to corresponding mouse sequence) and is non cross-reactive with Rac1, Rac2 or other Ras superfamily GTP binding proteins. Therefore, this result confirmed that the cDNA we are reporting is most likely the homologue of the GTPase human Cdc42.

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