ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice

Osamu Nakagawa[a,b], Kazuko Fujisawa[a], Toshimasa Ishizaki[a], Yuji Saito[a], Kazuwa Nakao[b], Shuh Narumiya[a,*]

[a]*Department of Pharmacology, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606, Japan*
[b]*Department of Medicine and Clinical Science, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606, Japan*

Received 3 June 1996

Abstract **We recently identified a novel human protein kinase, p160 ROCK, as a putative downstream target of the small GTPase Rho. Using the human ROCK cDNA as a probe, we isolated cDNA of two distinct, highly related sequences from mouse libraries. One encoded a mouse counterpart of human ROCK (ROCK-I), and the other encoded a novel ROCK-related kinase (ROCK-II). Like ROCK/ROCK-I, ROCK-II also bound to GTP-Rho selectively. ROCK-I mRNA was ubiquitously expressed except in the brain and muscle, whereas ROCK-II mRNA was expressed abundantly in the brain, muscle, heart, lung and placenta. These results suggest that at least two ROCK isoforms are present in a single species and play distinct roles in Rho-mediated signalling pathways.**

Key words: Rho GTPase; Signal transduction; Protein serine/threonine kinase; cDNA cloning; Gene expression

1. Introduction

The small GTPase Rho controls various cellular processes by shuttling between the inactive GDP-bound form and the active GTP-bound form. Rho is a key regulator of stimulus-evoked cell adhesion and motility, enhancement of contractile responses, cytokinesis and transcriptional regulation through the activation of serum response factor [1]. Rho apparently mediates multiple intracellular signalling pathways, and is likely to fulfil these actions by activating various downstream targets. We and others have demonstrated that protein phosphorylation by tyrosine kinases and serine/threonine kinases is involved in Rho-activated signalling [2–5]. The direct target molecules of Rho, however, remained unclarified. Using the yeast two hybrid system with Val^{14}-Rho as a bait or using the ligand overlay assay with [^{35}S]GTPγS-RhoA, we recently identified five putative target molecules. They can be classified into two groups. One includes a serine/threonine kinase PKN, and two novel non-kinase proteins, rhophilin and rhotekin [6,7]. These molecules possess a homologous Rho-binding motif of about 70 amino acids in length. The other is composed of a novel non-kinase molecule, citron, and a newly identified kinase, p160 ROCK [8,9]. They show Rho-binding sequences different from the motif seen in the first group and share general structural characteristics.

p160 ROCK is a protein serine/threonine kinase which has a kinase domain in the amino-terminus, an about 600 amino-acid-long coiled-coil forming region in the middle, and a pleckstrin homology region and a Cys-rich zinc finger-like motif in the carboxyl-terminus. The kinase domain of ROCK shows a significant sequence homology to myotonic dystrophy protein kinase (MD-PK) [9,10]. In this study, we screened mouse cDNA libraries using human p160 ROCK cDNA as a probe, and identified two related protein kinases. Our results indicate that ROCK protein kinases constitute a distinct kinase family composed of at least two isoforms.

2. Materials and methods

2.1. Isolation and sequencing of mouse ROCK-I and ROCK-II

Two cDNA fragments of human p160 ROCK were prepared as probes. The cDNA fragment coding for the amino-terminal kinase domain of human p160 ROCK (nucleotides 220–999 in the cDNA sequence U43195 in GenBank/EMBL Data Bank) was amplified by PCR using the cDNA fragment of human p160 ROCK as a template [9]. The *Spe*I fragment of human p160 ROCK cDNA encoding the carboxyl region (nucleotide 1094–4057 in U43195) was also prepared as a probe. These two probes were used to isolate cDNA fragments that cover the entire region of mouse ROCK. cDNA prepared from mouse embryo mRNA (E11.5 days) was constructed in bacteriophage λgt10 as previously described [11]. Approx. 5×10^5 plaques each from this mouse embryo cDNA library and from the mouse heart library in λgt10 (Clontech) were transferred to nylon membranes (Colony Plaque Screen, Du Pont NEN). Hybridization was carried out at 55°C in a solution containing 1 M sodium chloride, 1% SDS, 50 mM Tris-Cl (pH 7.5), 200 μg/ml heat-denatured salmon sperm DNA and ^{32}P-labelled probes. The membranes were washed serially, with a final wash in 2×SSC, 0.1% SDS at 55°C. The positive clones were purified, and restriction fragments of the cDNA inserts were subcloned into pBluescript SK(+). DNA sequencing was performed by the dideoxy-mediated chain termination method, using an ALFred automated DNA sequencer system (Pharmacia). The sequences were confirmed by reading both DNA strands.

2.2. Ligand overlay assay

The *Hinc*II-*Xba*I fragment of clone no. 87 corresponding to nucleotide 2452–3870 of mouse p160 ROCK-II (U58513 in GenBank/EMBL Data Bank) was subcloned to the plasmid pQE (pQE87HX). *E. coli* JM109 (Toyobo) was transformed with pQE87HX and was grown to an A_{600} of 0.8. After the addition of isopropyl β-D-thiogalactoside, the culture was continued for another 24 h at 20°C. Cells were lysed with 8 M urea containing 0.1 M sodium phosphate and 10 mM Tris-Cl (pH 8.0) at 25°C for 1 h. After centrifugation at 12000×*g* for 10 min, the supernatant was recovered and incubated with Ni-NTA resin (Qiagen) at 25°C for 1 h. The resin was washed with 8 M urea containing 0.1 M sodium phosphate and 10 mM Tris-Cl (pH 6.3), and boiled in Laemmli sample buffer for 5 min. The extracted His-tagged proteins were subjected to SDS-PAGE on 18% polyacrylamide gels, transferred onto nitrocellulose membranes (Schleicher & Schuell), de-

*Corresponding author. Fax: (81) (75) 753-4693.

Abbreviations: ROCK, Rho-associated coiled-coil forming kinase; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GTPγS, guanosine 5′-(γ-thio)triphosphate; MD-PK, myotonic dystrophy protein kinase; GDPβS, guanosine 5′-(β-thio)diphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

PII S0014-5793(96)00811-3

natured and renatured on the membranes. Recombinant human RhoA/GST fusion protein was prepared and loaded with either [^{35}S]GTPγS or [^{35}S]GDPβS, as previously described [9]. The membranes were then incubated with 8.1 nM ^{35}S-labelled Rho in the overlay assay buffer. The membranes were washed as previously described [9], and exposed to X-ray film.

2.3. Probe preparation and Northern blotting analyses

cDNA probes for Northern blotting analyses were prepared by PCR using isolated cDNA clones as templates. To prepare cDNA fragments corresponding to the kinase domain [12] (Fig. 1), oligonucleotide primers were synthesized: 5′-TAT GAA GTG GTA AAG GTA ATC GGC-3′ (sense for ROCK-I), 5′-GAA GAA GAG ATG ACG TTT GAT TTC-3′ (antisense for ROCK-I), 5′-TAT GAT GTT GTA AAA GTT ATT GGA-3′ (sense for ROCK-II) and 5′-AAA GAA AGG ATG CTG TTT GAT TTC-3′ (antisense for ROCK-II). PCR was performed under standard conditions [13]. Amplified 789 bp fragments were subcloned into the plasmid pCR2.1 (Invitrogen), and the sequences of the inserts were confirmed. Rat GAPDH cDNA fragment [14] was used as an internal control. These probes were labelled by the random priming method with [α-^{32}P]dCTP [13]. Specific activity was approx. 1×10^9 cpm/μg DNA. Total RNA was extracted from various tissues obtained from 14-week-old male BALB/c mice, whole embryos (E10 and E17 days) and the placenta (E17 days), using the AGPC method [13]. Poly(A)$^+$ RNA was purified using an oligo(dT) latex (oligoTEX dT30 Super, Daiichi Chemical). 3 μg aliquots of poly(A)$^+$ RNA were size-fractionated by electrophoresis on 1.0% agarose-formaldehyde gels and transferred to Biodyne A nylon membranes (Pall). The membranes were prehybridized at 42°C for 12 h in a solution containing 50% formamide, 5×SSPE, 5×Denhardt's solution, 0.1% SDS, and 100 μg/ml heat-denatured salmon sperm DNA. Hybridization was performed in the same solution including heat-denatured radioprobes at 42°C for 24 h. The membranes were washed serially, with a final wash in 0.1×SSC, 0.1% SDS at 65°C, and subjected to analysis using a BAS 2000 Bioimage analyzer (Fuji Film).

3. Results

3.1. cDNA cloning and characterization of mouse p160 ROCK-I and ROCK-II

We screened mouse embryo and heart libraries using human p160 ROCK cDNA as a probe. Two cDNAs were used as probes. One corresponded to the amino-terminal kinase domain of human p160 ROCK, and the other to the coiled-coil forming region and carboxyl-terminal region. Hybridization was separately performed using each probe. More than 100 positive clones were obtained by this procedure. Sequence analyses were first performed on clones hybridized with the probe for the kinase domain, and then extended to clones showing overlapping sequences. Two composite cDNA sequences were obtained, and they encoded related but distinct protein kinases.

A 6181-nucleotide cDNA sequence identified by the analysis of five clones, nos. 2, 7, 8, 13 and 119, contained a 4062-nucleotide open reading frame, which encoded a 1354-amino-acid protein with a relative molecular mass of 158 168 Da (Fig. 1A). The putative initiation site was preceded by an in-frame termination codon. The nucleotide sequence of the open reading frame and deduced amino acid sequence were highly homologous to those of human p160 ROCK (92.0 and 96.5%, respectively). In addition, functional domains observed in human p160 ROCK were conserved in this mouse sequence. A protein serine/threonine kinase domain was present in the amino-terminal region (amino acids 76–338) [12], and a coiled-coil forming region predicted by the algorithm of Lupas et al. [15] (amino acids 460–1068) followed. A pleckstrin homology domain [16] and a cysteine-rich zinc finger motif [17] exist in the carboxyl-terminal region (Fig. 1B). We, therefore, concluded that this represents a mouse counterpart of human p160 ROCK, and named it mouse p160 ROCK-I.

The second sequence identified by clones nos. 22, 24, 87 and 115 was 5743 nucleotide long, and contained a 4164-nucleotide open reading frame encoding 1388-amino-acid protein with a relative molecular mass of 160 583 Da (Fig. 1A). Its putative initiation site was also preceded by an in-frame termination codon. The nucleotide and amino acid sequence homologies to human p160 ROCK were 60.9 and 65.3%, respectively. While they are much lower than those of mouse ROCK-I, all the functional domains present in human p160 ROCK were also conserved in this molecule (Fig. 1B). The kinase domains showed particularly high homology between mouse ROCK-I and ROCK-II (92.0% in the amino acid sequence). These results indicate that this molecule is a protein kinase closely related to mouse ROCK-I, and we designated it mouse p160 ROCK-II.

We previously demonstrated that human p160 ROCK specifically binds to RhoA in a GTP-dependent manner [9]. Deletion and mutation analyses coupled with the ligand overlay assay and the yeast two hybrid assay identified amino acids 934–1015 of human p160 ROCK as the Rho-binding domain (Fujisawa et al., unpublished observation). This part of the sequence was highly conserved in mouse ROCK-I, whereas the conservation in ROCK-II was approx. 55%. In order to demonstrate the association of Rho with ROCK-II, we prepared a recombinant protein containing the corresponding region of mouse ROCK-II in *E. coli*, and examined its binding to human RhoA/GST fusion protein loaded with [^{35}S]GTPγS or [^{35}S]GDPβS. As seen in Fig. 2, this truncated protein of mouse ROCK-II selectively bound to the GTP-bound form of RhoA.

3.2. Northern blotting analysis of mouse ROCK-I and ROCK-II

Gene expression of ROCK-I and ROCK-II in various mouse tissues was examined by Northern blotting analyses using cDNA corresponding to the kinase domains as probes. A 6.6 kb mRNA band of ROCK-I was detected in all the tissues and whole fetuses (Fig. 3A). Mouse ROCK-I mRNA was abundantly expressed in the heart, lung, liver, stomach, spleen, kidney, testis, placenta and fetus, but much less in the brain and skeletal muscle. An additional transcript of 6.0 kb was observed in the testis. Expression of mRNA for mouse ROCK-II showed distinct pattern from that of mouse ROCK-I (Fig. 3B). Three major transcripts of 7.6, 6.6 and 5.6 kb were detected. Expression of mouse ROCK-II mRNA was high in the brain, heart, lung, muscle, placenta and fetus, and was relatively low in the liver, stomach, spleen, kidney and testis.

4. Discussion

In this study, two protein kinases, ROCK-I and ROCK-II, were isolated as mouse homologues of human p160 ROCK. The alignments of their functional domains are identical, as presented in Fig. 1B and as reported previously [9]. The amino acid sequence of the kinase domain of mouse ROCK-I is completely identical to that of human p160 ROCK, which shows serine/threonine kinase activity in vitro and in vivo [9]. The amino acid sequences of mouse ROCK-I and ROCK-II are 92% homologous and well consistent with the

A

```
ROCK-I                                MSTGDSFETRFEKIDNLLRDPKSEVNSDCLLDGLDALVYDLDFPALRKNKNIDNFLSRYKDTINKIRDLRMKAEDYEVVKVIGR   84
ROCK-II   MSRPPPTGKMPGAPEAAPGDGAGAGRQRKLEALIRDPRSPINVESLLDGLNSLVLDLDFPALRKNKNIDNFLNRYEKIVKKIRGLQMKAEDYDVVKVIGR   100
ROCK-I    GAFGEVQLVRHKSTRKVYAMKLLSKFEMIKRSDSAFFWEERDIMAFANSPWVVQLFYAFQDDRYLYMVMEYMPGGDLVNLMSNYDVPEKWARFYTAEVVL   184
ROCK-II   GAFGEVQLVRHKASQKVYAMKLLSKFEMIKRSDSAFFWEERDIMAFANSPWVVQLFCAFQDDRYLYMVMEYMPGGDLVNLMSNYDVPEKWAKFYTAEVVL   200
ROCK-I    ALDAIHSMGFIHRDVKPDNMLLDKSGHLKLADFGTCMKMNKEGMVRCDTAVGTPDYISPEVLKSQGGDGYYGRECDWWSVGVFLYEMLVGDTPFYADSLV   284
ROCK-II   ALDAIHSMGLIHRDVKPDNMLLDKHGHLKLADFGTCMKMDETGMVHCDTAVGTPDYISPEVLKSQGGDGYYGRECDWWSVGVFLFEMLVGDTPFYADSLV   300
ROCK-I    GTYSKIMNHKNSLTFPDDNDISKEAKNLICAFLTDREVRLGRNGVEEIKRHLFFKNDQWAWETLRDTVAPVVPDLSSDIDTSNFDDLEEDKGDEETFPIP   384
ROCK-II   GTYSKIMDHKNSLCFPEDTEISKHAKNLICAFLTDREVRLGRNGVEEIKQHPFFKNDQWNWDNIRETAAPVVPELSSDIDSSNFDDIEDDKGDVETFPIP   400
ROCK-I    KAFVGNQLPFVGFTYYSNRRYLPSANASENRSSSNVDKSLQ-ESLQKTIYKLEEQLHNEMQLKDEMEQKCRTSNLKLDKIMKELDEEGNQRRNLESAVSQ   483
ROCK-II   KAFVGNQLPFIGFTYFRENLLLSDSPPCRENDAIQTRKSEESQEIQKKLYALEEHLSSEVQAKEELEQKCKSINTRLEKTAKELEEEITLRKSVESTLRQ   500
ROCK-I    IEKEKMLLQHRINEYQRKVEQENEKRRNIENEVSTLKDQLEDLRKASQTSQLANEKLTQLQKQLEEANDLLRTESDTAVRLRKSHTEMSKSISQLESLNR   583
ROCK-II   LEREKALLQHKNAEYQRKADHEADKKRNLENDVNSLKDQLEDLKKRNQSSQISTEKVNQLQKQLDEANALLRTESDTAARLRKTQAESSKQIQQLESNNR   600
ROCK-I    ELQERNRILENSKSQADKDYYQLQAVLEAERRDRGHDSEMIGDLQARITSLQEEVKHLKHNLERVEGERKEAQDMLNHSEKEKNNLEIDLNYKLKSIQQR   683
ROCK-II   DLQDKNCLLETAKLKLEKEFINLQSALESERRDRTHGSEIINDLQGRISGLEEDLKTGKALLAKVELEKRQLQEKLTDLEKEKSNMEIDMTYQLKVIQQS   700
ROCK-I    LEQEVNEHKVTKARLTDKH---QSIEEAKSVAMCEMEKKLKEERERAREKAENRVVETEKQCSMLDVDLKQSQQKLEHLTENKERMEDEVKNLALQLEQES   780
ROCK-II   LEQEEAEHKTTKARLADKNKIYESIEEAKSEAMKEMEKKLLEERSLKQKVENLLLEAEKRCSILDCDLKQSQQKLNELLKQKDVLNEDVRNLTLKIEQET   800
ROCK-I    NKRLLLQNELKTQAFEADNLKGLEKQMKQEINTLLEAKRLLEFELAQLTKQYRGNEGQMRELQDQLEAEQYFSTLYKTQVKELKEEIEEKNR---ENLRK   877
ROCK-II   QKRCLMQNDLKMQTQQVNTLKMSEKQIKQENNHLMEMKMNLEKQNTELRKERQDADGQMKELQDQLEAEQYFSTLYKTQVRELKEENEEKTKLCKELQQK   900
ROCK-I    IQELQSEKETLSTQLDLAETKAESEQLARGILEEQYFELTQE-------SKKAASRNRQEITDKDHTVSRLEETNSVLTKDIEMLRKENEELNERMRTAE   970
ROCK-II   KQDLQDERDSLAAQLEITLTKADSEQLARSIAEEQYSDLEKEKIMKELEIKEMMARHKQELTEKDTTIASLEETNRTLTSDVANLANEKEELNNKLKDSQ   1000
ROCK-I    EEY-KLKKEE-EINNLKAAFEKNISTERTLKTQAVNKLAEIMNRKDFKIDRKKANTQDLRKKEKENRKLQLELNQEREKFNQMVVKHQKELNDMQAQLVE   1068
ROCK-II   EQLSKLKDEEMSAAAIKAQFEKQLLNERTLKTQAVNKLAEIMNRKE---PVKRGSDTDVRRKEKENRKLHMELKSEREKLTQQMIKYQKELNEMQAQIAE   1097
ROCK-I    ECTHRNELQMQLASKESDIEQLRAKL--LDLS-DSTSVASFPSADETDGNLPESRIEGWLSVPNRGNIKRYGWKKQYVVVSSKKILFYNDEQDKEQSSPS   1165
ROCK-II   ESQIRIELQMTLDSKDSDIEQLRSQLQALHIGMDSSSIGSGPGDAEPDDGFPESRLEGWLSLPVRNNTKKFGWVKKYVIVSSKKILFYDSEQDKEQSNPY   1197
ROCK-I    MVLDIDKLFHVRPVTQGDVYRAETEEIPKIFQILYANEGECRKDIEVEPVQQGEKTNFQNHKGHEFIPTLYHFPANCEACAKPLWHVFKPPPALECRRCH   1265
ROCK-II   MVLDIDKLFHVRPVTQTDVYRADAKEIPRIFQILYANEGESKKEPEFPVEPVGEKSNYICHKGHEFIPTLYHFPTNCEACMKPLWHMFKPPPALECRRCH   1297
ROCK-I    VKCHRDHLDKKEDLISPCKVSYDVTSARDMLLLACSQDEQKKWVTHLVKKIPKNPP--SGFVRASPRTLSTRSTANQSFRKVVKNTSGKTS           1354
ROCK-II   IKCHKDHMDKKEEIIAPCKVYYDISSAKNLLLLANSTEEQQKWVSRLVKKIPKKPPAPDPFARSSPRT-SMKIQQNQSIRRPSRQLAPNKPS          1388
```

B

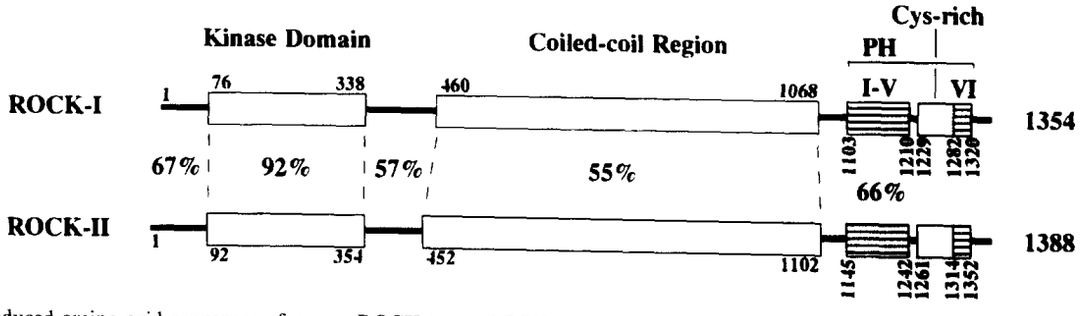

Fig. 1. Deduced amino acid sequences of mouse ROCK-I and ROCK-II (A) and schematic representation of their predicted functional domains (B). (A) Deduced amino acid sequences of mouse ROCK-I and ROCK-II are aligned according to their homology. Amino acids are numbered at the end of each line. Horizontal bars in the sequences represent insertions to maximize the homology of the aligned sequences. Vertical lines represent identical amino acid residues. The 16th codon (GCC, alanine), identified in clone no. 22 and shown here, was substituted to (ACC, threonine) in two other clones of ROCK-II (nos. 115, 116). The accession numbers of the cDNA sequences of mouse ROCK-I and ROCK-II in the GenBank/EMBL Data Bank are U58512 and U58513, respectively. (B) Predicted functional domains are shown by boxes. Kinase domain, protein serine/threonine kinase domain; coiled-coil region, amphipathic α-helix (coiled-coil forming region); PH (I–VI), pleckstrin homology domain (I–VI); Cys-rich, cysteine-rich zinc finger-like domain. Numbers represent those of amino acid residues. The Rho-binding domain identified in human p160 ROCK corresponds to amino acids 934–1015 of mouse ROCK-I.

motif conserved among various protein serine/threonine kinases [12]. In addition, the amino acid sequence of the Rho-binding domain of human ROCK is well conserved in mouse ROCK-I, and the recombinant ROCK-II protein containing the corresponding region showed GTP-dependent binding to RhoA. These results suggest that mouse ROCK-I and ROCK-II act as protein serine/threonine kinases downstream of Rho in cellular signalling.

Among the functional domains, the α-helical, coiled-coil forming structure is most diverse between ROCK-I and

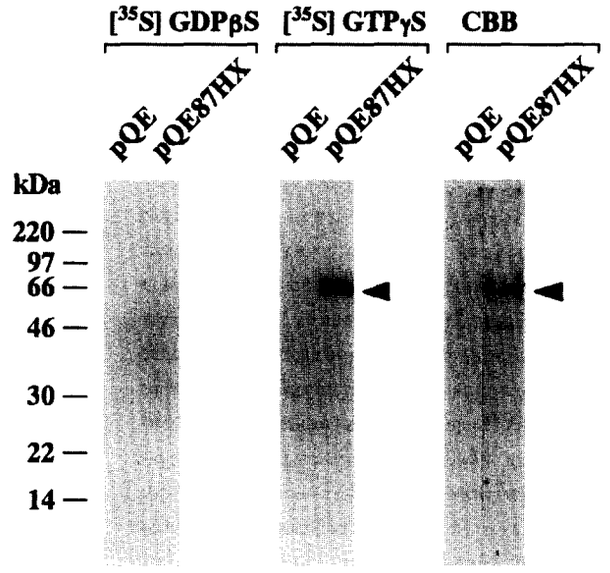

Fig. 2. GTP-dependent binding of recombinant RhoA to mouse ROCK-II fragment. Fragment of mouse ROCK-II (amino acids 818–1290) was prepared as a His-tagged protein, and subjected to ligand overlay assay using [^{35}S]GDPβS- or [^{35}S]GTPγS-loaded GST-human RhoA. pQE, pQE vector alone; pQE87HX, pQE containing the *Hin*cII/*Xba*I fragment of no. 87 clone of mouse ROCK-II cDNA; CBB, Coomassie brilliant blue staining. Positions of molecular size markers are shown on the left (in kDa).

ROCK-II. A coiled-coil forming region is supposed to dimerize or interact with other amphipathic α-helical proteins, which leads to functional regulation and intracellular localization of these proteins [18]. The difference in the coiled-coil forming sequence of ROCK-I and ROCK-II suggests a possibility that they may have different localization and play distinct roles in the cell. MD-PK, which contains a kinase domain and a coiled-coil forming region homologous to ROCK [9,10], is specifically localized to the myotendenous junction of skeletal muscle, the dense plaques of smooth muscle and the intercalated disc of cardiac muscle [19], in which muscle fibers fuse to the membranes and many focal adhesion proteins accumulate. ROCK may also be present in related intracellular compartments and be involved in Rho-mediated cytoskeletal regulation.

Recently, Leung et al. [20] and Matsui et al. [21] identified rat and bovine protein kinases as putative Rho targets, which are homologous to human p160 ROCK. The sequences of these protein kinases show much higher homology with mouse ROCK-II than mouse ROCK-I, although the reported rat sequence does not contain the segment corresponding to the amino-terminal region of ROCK-II. These rat and bovine kinases are, therefore, counterparts of mouse ROCK-II, while human p160 ROCK is a counterpart of mouse ROCK-I. In addition, Northern blotting analysis on the human tissue demonstrated that the probes for ROCK-I or ROCK-II detected distinct transcripts with different sizes (Nakagawa et al., unpublished observations). Taken together, we can conclude that there are at least two ROCK isoforms in a single species.

As presented in Fig. 3, the gene expression of mouse ROCK-I and ROCK-II is differentially regulated in mouse tissues. A single mRNA band of ROCK-I was detected except in the testis, while three major transcripts of ROCK-II were expressed. It remains an open question whether this is due to alternative splicing or multiplicity of polyadenylation sites. As the length and radioactivity of the cDNA probes were approximately the same, the mRNA abundance of ROCK-I and ROCK-II could be compared in each tissue. ROCK-I mRNA is preferably expressed in the liver, spleen, kidney and testis. In contrast, ROCK-II mRNA is predominant in

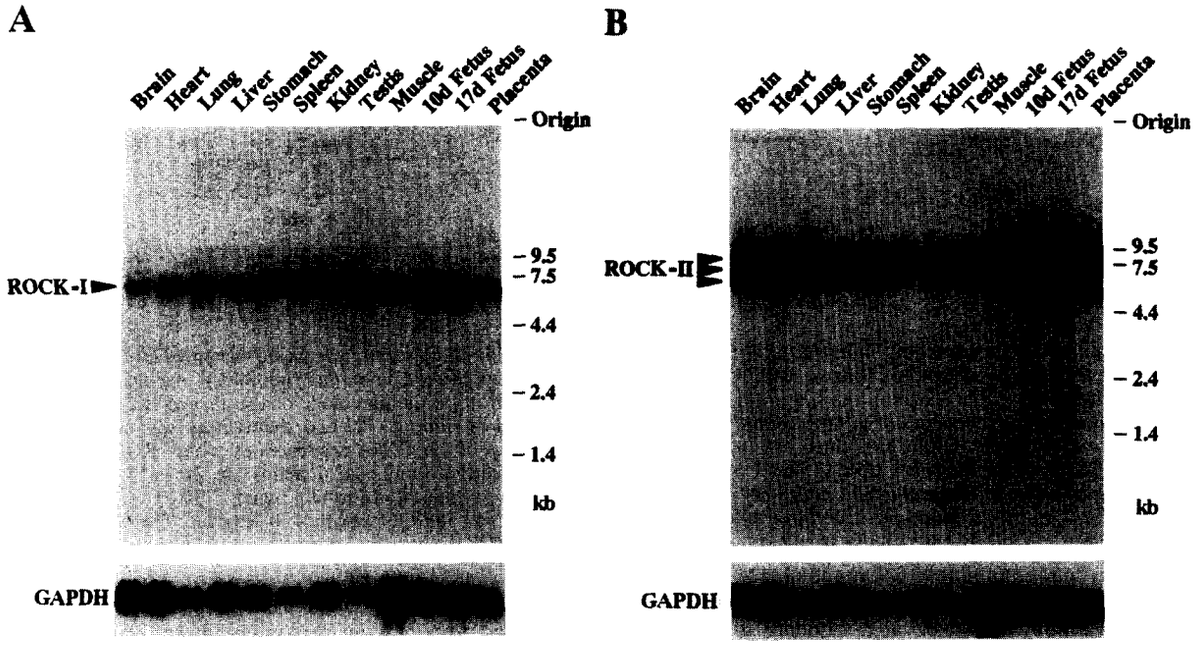

Fig. 3. Tissue distribution of mRNA for mouse ROCK-I and ROCK-II. Northern blotting analyses of mouse ROCK-I (A) and mouse ROCK-II (B) were performed. Detected transcripts are shown by arrows, and positions of RNA size markers are shown on the right (in kb). Rehybridization with a rat GAPDH cDNA probe was carried out, and results are shown as an internal control at the bottom.

other tissues, especially in the brain and skeletal muscle. These findings raise the possibility that ROCK-I and ROCK-II may play distinct roles in a tissue-specific manner.

In summary, we have identified two isoforms of Rho-associated protein kinase, ROCK. The present study will facilitate our understanding of the molecular mechanisms of multiple Rho functions.

Acknowledgements: O.N. is supported by a research fellowship from the Japan Society for the Promotion of Science. We thank Drs. A. Kakizuka, P. Madaule, M. Maekawa, G. Watanabe, N. Watanabe, A. Fujita, and M. Naito for valuable advice. We also acknowledge secretarial and technical assistance by Ms. K. Okuyama and H. Fuyuhiro. This work was supported in part by Grant-in-aid for Specially Promoted Research and Grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and by a grant from the Human Frontier Science Program.

References

[1] Narumiya, S. (1996) J. Biochem., in press.
[2] Kumagai, N., Morii, N., Fujisawa, K., Nemoto, Y. and Narumiya, S. (1993) J. Biol. Chem. 268, 24535–24538.
[3] Ridley, A.J. and Hall, A. (1992) EMBO J. 13, 2600–2610.
[4] Kumagai, N., Morii, N., Ishizaki, T., Watanabe, N., Fujisawa, K., Saito, Y. and Narumiya, S. (1995) FEBS Lett. 366, 11–16.
[5] Ridley, A.J. and Hall, A. (1992) Cell 70, 389–399.
[6] Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A. and Narumiya, S. (1996) Science 271, 645–648.
[7] Reid, T., Furuyashiki, T., Ishizaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madaule, P. and Narumiya, S. (1996) J. Biol. Chem., in press.
[8] Madaule, P., Furuyashiki, T., Reid, T., Ishizaki, T., Watanabe, G., Morii, N. and Narumiya, S. (1995) FEBS Lett. 377, 243–248.
[9] Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito,Y., Kakizuka, A., Morii, N. and Narumiya, S. (1996) EMBO J. 15, 1885–1893.
[10] Brook, J.D., McCurrach, M.E., Harley, H.G., Buckler, A.J., Church, D., Aburatani, H., Hunter, K., Stanton, V.P., Thirion, J.-P., Hudson, T., Sohn, R., Zemelman, B., Snell, R.G., Rundle, S.A., Crow, S., Davies, J., Shelbourne, P., Buxton, J., Jones, C., Juvonen, V., Johnson, K., Harper, P.S., Shaw, D.J. and Housman, D.E. (1992) Cell 68, 799–808.
[11] Kakizuka, A., Yu, R.T., Evans, R.M. and Umesono, K. (1993) in: cDNA Library Construction (Stein, C.D. and Holland, P.W.H. eds.) pp. 223–232, Essential Developmental Biology: A Practical Approach, IRL Press, Oxford.
[12] Hanks, S.T. and Quinn, A.M. (1991) Methods Enzymol. 200, 38–62.
[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[14] Tso, J.Y., Sun, X.-H., Kao, T., Reece, K.S. and Wu, R. (1985) Nucleic Acids Res. 13, 2485–2502.
[15] Lupas, A., Van Dyke, M. and Stock, J. (1991) Science 252, 1162–1164.
[16] Musacchio, A., Gibson, T., Rice, P., Thompson, J. and Saraste, M. (1993) Trends Biochem. Sci. 18, 343–348.
[17] Hubbard, S.R., Bishop, W.R., Kirschmeier, P., George, S.J., Cramer, S.P. and Hendrickson, W.A. (1991) Science 254, 1776–1779.
[18] Cohen, C. and Parry, D.A.D. (1986) Trends Biochem. Sci. 11, 245–248.
[19] van der Ven, P.F.M., Jansen, G., van Kuppevelt, T.H.M.S.M., Perryman, M.B., Lupa, M., Dunne, P.M., ter Laak, H.J., Jap, P.H.K., Veerkamp, J.H., Epstein, H.F. and Wieringa, B. (1983) Hum. Mol. Genet. 2, 1889–1894.
[20] Leung, T., Manser, E., Tan, L. and Lim, L. (1995) J. Biol. Chem. 270, 29051–29054.
[21] Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) EMBO J. 15, 2208–2216.