Identification and characterization of *Drosophila* homolog of Rho-kinase

Keywords: Rho effector, actin cytoskeleton, morphogenesis, low stringency PCR, two-hybrid analysis, *in vitro* kinase assay

Tomoaki Mizuno[1], Mutsuki Amano[2], Kozo Kaibuchi[2] and

Yasuyoshi Nishida[1]*

[1] *Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan.*

[2] *Division of Signal Transduction, Nara Institute of Science and Technology, Ikoma 630-0101, Japan.*

* Corresponding author: Yasuyoshi Nishida

Division of Biological Science

Graduate School of Science, Nagoya University

Chikusa-ku, Nagoya 464-8602

Japan

Tel: +81-52-789-2472

FAX: +81-52-789-2511

e-mail: nishida@bio.nagoya-u.ac.jp

Abbreviations: bp, base pair(s); C, carboxy; kb, kilobase(s); *DRhk*, *DRho-kinase*; EST, expressed sequence tag; MBP, myelin basic protein; MBS, myosin-binding subunit of myosin phosphatase; MLC, myosin light chain; N, amino; PCR, polymerase chain reaction; PH, pleckstrin homology; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Abstract

The Rho family of small GTPases and their associated regulators and targets are essential mediators of diverse morphogenetic events in development. Mammalian Rho-kinase/ROKα, one of the targets of Rho, has been shown to bind to Rho in GTP-bound form and to phosphorylate the myosin light chain (MLC) and the myosin binding subunit (MBS) of myosin phosphatase, resulting in the activation of myosin. Thus, Rho-kinase/ROKα has been suggested to play essential roles in the formation of stress fibers and focal adhesions. We have identified the *Drosophila* homolog of Rho-kinase/ROKα, DRho-kinase, which has conserved the basic structural feature of Rho-kinase/ROKα consisting of the N-terminal kinase, central coiled-coil and C-terminal pleckstrin homology (PH) domains. A two-hybrid analysis demonstrated that DRho-kinase interacts with the GTP-bound form of the *Drosophila* Rho, Drho1, at the conserved Rho-binding site. DRho-kinase can phosphorylate MLC and MBS, preferable substrates for bovine Rho-kinase, *in vitro*. *DRho-kinase* is ubiquitously expressed throughout development, in a pattern essentially identical to that of *Drho1*. These results suggest that DRho-kinase is an effector of Drho1.

1. Introduction

The Rho family of small GTPases control a diverse array of morphogenetic processes involving the dynamic remodeling of the actin cytoskeleton by acting as molecular switches which interconvert between two states; the active, GTP-bound and the inactive, GDP-bound states (Bouren *et al*., 1991; Kaibuchi *et al*., 1999). The Rho family has been strongly conserved during evolution and consists of the Rho, Rac and

Cdc42 subfamilies. Each of the Rho family members participates in a distinct pattern of actin reorganization, and Rho, Rac and Cdc42 underlie the formation of stress fibers, lamellipodia and fillopodia, respectively, in mammalian fibroblasts (Kozma *et al*., 1995; Nobes *et al*., 1995). Although members of the Rho subfamily were initially characterized as regulators of cytoskeletal organization in response to extracellular signals, it is now known that the subfamily is involved in several other cellular processes such as transcriptional regulation and control of cell growth (Kaibuchi *et al*., 1999). Thus, Rho appears to regulate diverse cellular functions through multiple target molecules.

A number of target proteins to which Rho binds in a GTP-dependent manner have been identified, including Rho-kinase/ROK/ROCK, citron, PKN, Rhotekin and p140mDia (reviewed by Kaibuchi *et al*., 1999). Of particular interest is the serine/threonine kinase, Rho-kinase/ROKα/ROCK-II, one of the isoforms of Rho-kinase/ROK/ROCK. It has been demonstrated that expression of the full-length Rho-kinase/ROKα, or its N-terminal fragment containing the kinase domain, promotes the formation of stress fibers and focal adhesions (Leung *et al*., 1995; Amano *et al*., 1997). On the contrary, expression of a kinase-defective mutant protein or C-terminal fragments resulted in the disassembly of stress fibers and focal adhesions (Leung *et al*., 1995; Amano *et al*., 1997). Rho-kinase/ROKα has been shown to phosphorylate the myosin light chain (MLC) of myosin II at the same site that is phosphorylated by MLC kinase (Amano *et al*., 1996b). Furthermore, Rho-kinase/ROKα phosphorylates the myosin-binding subunit (MBS) of myosin phosphatase, resulting in a decrease in the phosphatase activity (Kimura *et al*., 1996). Thus, through these dual mechanisms, Rho-

kinase/ROKα causes the accumulation of the phosphorylated form of MLC, which stimulates myosin to bind to actin filament and results in the formation of stress fibers and focal adhesions. These findings suggested that Rho-kinase/ROKα plays an essential role in the regulation of the actin cytoskeletal functions.

At least five Rho family members, Drho1, Drac1/DRacA, Drac2, Drac3 and Dcdc42, have been identified in *Drosophila*, and expression of their dominant-negative or constitutive-active forms in the fly suggested that the Rho, Rac and Cdc42 homologs control different actin-dependent processes during developement, such as cell shape changes, myoblast fusion, axon outgrowth and cytokinesis (Harden *et al*., 1995; Hariharan *et al*., 1995; Eaton *et al*., 1995; Murphy *et al*., 1996; Crawford *et al*., 1998). Drho1 has been characterized genetically, and its functions in diverse morphogenetic processes have been suggested. Embryos homozygous for the null alleles of *Drho1* exhibit an 'anterior open' phenotype, suggesting the role of Drho1 in morphological movements or cell polarity in the embryonic epidermis (Strutt *et al*., 1997). It has been also demonstrated that Drho1 is required in the generation of tissue polarity in the wing and the compound eye and acts downstream of Frizzled (Fz), a serpentine receptor (Strutt *et al*., 1997). Furthermore, analyses of the loss-of-function mutations of *DRhoGEF2*, which encodes a Drho1-specific guanine nucleotide exchange factor, suggested that Drho1 is essential for the coordination of cell shape changes during gastrulation (Barrett *et al*., 1997; Häcker and Perrimon, 1998). However, little is known about the effector molecules of Drho1. To help elucidate the molecular mechanisms involved in the Rho-mediated cytoskeletal functions in development, we have identified and characterized the *Droshophila* homolog of Rho-kinase.

2. Materials and methods

2. 1. Degenerate PCR amplification

Degenerate PCR primers were designed based on the amino acid sequences in the conserved kinase domain (Fig. 1C). The sequences are 5'-AAYTAYGAYGTNCCNGARAARTGGA-3' and 5'-RTTYTTRTGRTTCATDATYTT-3'. Adult fly DNA was used as a template. A low stringency PCR was performed according to the following protocol: denaturation at 94°C for 1 min followed by chain reactions for 10 cycles of 94°C for 20 sec, 37°C for 1 min and 68°C for 2 min, then chain reactions for a further 20 cycles of 94°C for 20 sec, 52°C for 1 min and 68°C for 2 min. The PCR products were cloned into pGEM-T (Promega Corp.).

2. 2. cDNA cloning

An embryonic cDNA library (Umemiya *et al*., 1997) was screened with probes radiolabeled by the Multiple DNA Labeling System (Amersham Pharmacia Biotech). Phage DNAs were immobilized on Hybond-N membranes (Amersham Pharmacia Biotech) and hybridized for 12 hr at 65°C in the Rapid Hybridization Buffer (Amersham Pharmacia Biotech).

2. 3. Plasmid constructions

The *Drho1* cDNA was kindly provided by J. Settleman. EST clones made by the Berkeley *Drosophila* Genome Project were purchased from Research Genetics Inc. Modifications in *Drho1* or *DRho-kinase* cDNAs were made by oligonucleotide-directed

PCR mutagenesis. For two-hybrid analysis, the *Drho1* and *DRho-kinase* cDNA fragments were cloned in frame into pBTM116 and pACTII vectors, respectively, after relevant modifications (Fields *et al*., 1988; Vojtek *et al*., 1993). DRho-kinase was tagged with triplicate HA epitopes at its N-terminus by subcloning the cDNA fragment into the pcDNA3 expression vector (Invitrogen).

2. 4. Two-hybrid analysis

The *Saccharomyces cerevisiae* reporter strain L40 [*MAT**a***, *trp1*, *leu2*, *his3*, *LYS2*::(l*exAop*)$_4$-*HIS3*, *URA3*::(*lexAop*)$_8$-*LacZ*] was co-transformed with plasmids expressing the LexA-DRho-kinase and GAL4-DRho1 fusion proteins. The transformants were plated onto a synthetic medium lacking histidine and containing 10 mM 3-amino triazole and were incubated at 30°C for 48 to 72 hr. If the pairs of fusion proteins interact with each other, transformants can express the *HIS3* reporter gene and therefore can grow on the plate.

2. 5. Immunoprecipitation and immunoblotting

The human 293 cells were maintained in Dulbecco's Modified Eagle medium supplemented with fetal calf serum (10%) at 37°C under 5% CO_2. Cells were plated at a density of 1 x 10^6 cells per 10 cm dish and were transfected with a total of 10 μg DNA by the Ca^{2+} phosphate precipitation method. After 36 hr cultivation, cells were washed once with PBS, harvested and lysed in 0.3 ml of 0.5% Triton-X100 lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5

mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM PMSF and 20 μM aprotinin. Cellular debris was removed by centrifugation at 10,000 x g for 5 min, and proteins were immunoprecipitated with anti-HA antibody HA.11 (Babco) and Protein G-Sepharose (Amersham Pharmacia Biotech). For immunoblotting, the beads were boiled in SDS sample buffer and the eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto Hybond-P membranes (Amersham Pharmacia Biotech), which were then blotted with anti-HA antibody Y-11 (Santa Cruz Biotechnology Inc.). Bound antibody was visualized with horseradish peroxidase-conjugated anti-rabbit IgG antibody using the enhanced chemiluminescence (ECL) blotting system (Amersham Pharmacia Biotech).

2. 6. in vitro kinase assay

HA-DRho-kinase immunoprecipitated as above was incubated with 1.9 μg of purified chick MLC (Amano *et al*., 1996b) or 0.39 μg of recombinant MBS (Kimura *et al*., 1996) in 10 μl kinase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 1 mM EDTA, 1 mM Na_3VO_4, 0.4 mM PMSF, 1 mM ATP, 20 mM creatine phosphate and 5 μCi of [γ-^{32}P]-ATP (3000 Ci/mmol) at 25°C for 30 min. Samples were analyzed by SDS-PAGE and autoradiography.

2. 7. in situ hybridization

in situ hybridization to whole mount embryos and imaginal discs was performed using digoxigenin-labeled, anti-sense or sense RNA probes as described by Nikaido *et al*. (1997).

3. Results and discussion

3.1. Cloning of Drosophila *homolog of Rho-kinase*

The kinase domain sequence is highly conserved among the Rho-kinase/ROKα homologs (Fig. 1B; Leung *et al*., 1995; Ishizaki *et al*., 1996; Matsui *et al*., 1996; Nakagawa *et al*., 1996; Wissmann *et al*., 1997; Farah *et al*., 1998). To clone a *Drosophila* homolog of Rho-kinase/ROKα, degenerate oligonucleotide primers corresponding to the conserved sequences in the kinase domain were designed and used in low stringency polymerase chain reactions (PCR) with *Drosophila* genomic DNA as a template. The resulting PCR products of about 400 bp long were cloned and sequencing of five independent clones revealed an identical sequence in each. A BLAST search revealed that the sequence is highly related to the reported sequences for Rho-kinase/ROKα, but no corresponding *Drosophila* sequence was obtained. The cloned PCR fragment was used to probe an embryonic cDNA library, and seven clones were obtained. They provided a nucleotide sequence further to the 5' side than the previously obtained sequence of the PCR fragment. The sequence was used to search for EST clones made by the Berkeley *Drosophila* Genome Project, and four matching clones were identified; LD11891, LD15203, LD31145 and GM09611.

The longest EST clone, LD11891, is 6.3 kb long and contains the entire coding sequence as well as the 5'- and 3'-UTR, and poly-A sequences. The nucleotide

sequence predicted the primary structure of a protein of 1,390 amino acid residues with a calculated molecular mass of 160,325 (Fig. 1A). Comparison of the predicted amino acid sequence with the reported sequences revealed that the *Drosophila* protein closely resembles the Rho-kinase/ROKα sequences and exhibits the common structural feature of a Rho-kinase/ROKα that consists of the N-terminal kinase, central coiled-coil and the C-terminal pleckstrin-homology (PH) domains (Fig. 1B). The characteristic Cys-rich region inserted in the PH domain of Rho-kinase/ROKα is also conserved in DRho-kinase. The highest similarity was seen in the kinase domain and more than 70% of the residues are identical with bovine, rat and *Xenopus* Rho-kinases. The sequence in the kinase domain also resembles the Rho-kinase-related sequences of *C. elegans* LET-502, which lacks the C-terminal PH domain, and Gek, an effector of *Drosophila* Cdc42 (Fig. 1B and C; Luo *et al*., 1997). Furthermore, the *Drosophila* sequence showed a 43% overall identity with bovine Rho-kinase. The structural similarity clearly indicates that the cloned cDNA encodes a *Drosophila* homolog of Rho-kinase. Analysis of the Berkeley *Drosophila* Genome Database revealed that the *DRho-kinase* (*DRhk*) gene is partially contained in the genomic P1 clone, DS07054, which is located between *rudimentary* (*r*) and *inflated* (*if*) at the 15A1 region in the salivary gland chromosome map.

3. 2. Physical interaction of DRho-kinase with Drho1

The structural conservation suggested that DRho-kinase would be a target of the *Drosophila* Rho, Drho1, and the interaction of DRho-kinase with Drho1 was addressed by the yeast two-hybrid system. To facilitate the nuclear localization of the fusion

proteins between the LexA DNA-binding domain and Drho1, the four C-terminal amino acid residues of Drho1, Cys-Leu-Leu-Leu, responsible for lipid attachment and membrane targeting were deleted according to Ozaki *et al*. (1996) and Luo *et al*. (1997). Either Gly14 or Thr19 of Drho1 was further substituted with Val or Asn, respectively, to make Drho1 to remain in the active, GTP-bound or inactive, GDP-bound forms, respectively.

As shown in Fig. 2A, the reporter *HIS3* gene was induced only when the plasmid encoding the entire DRho-kinase fused to the GAL4 transcription activation domain was co-introduced with the plasmid expressing LexA-Drho1$^{V14\Delta C}$. No interaction was seen with the wild type or the inactive forms of Drho1. This result indicates that DRho-kinase interacts with Drho1 in the GTP form.

The Rho-binding site in the rat ROKα and bovine Rho-kinase lies in a 90 amino acid segment in the C-terminal region of the coiled-coil domain (Leung *et al*., 1995; Matsui *et al*. 1996; Fig. 1B). The sequence in the corresponding region of DRho-kinase showed considerable similarity to those of the mammalian Rho-kinases (Fig. 1D). Furthermore, the Asn and Lys residues in the Rho-binding site, which are essential for Rho-binding (Leung *at al*., 1996), are conserved in DRho-kinase (asterisks in Fig. 1D). To determine whether this sequence in DRho-kinase is responsible for the binding to Drho1, a construct expressing a fusion protein between the GAL4 activation domain and the fragment of DRho-kinase spanning from the amino acid residue 912 to 1109, which contains the entire conserved stretch, was constructed and tested for interaction with Drho1 by the two-hybrid system. As shown in Fig. 3B, the fragment binds to Drho1$^{V14\Delta C}$, and the interaction was abolished when the conserved Asn1017 and

Lys1018 were substituted with Thr residues (Fig. 2B). These results indicate that the GTP-bound Drho1 binds to DRho-kinase at the conserved Rho-binding site.

3. 3. Kinase activity of DRho-kinase

We examined the ability of DRho-kinase to phosphorylate vertebrate MLC and MBS, preferable substrates for bovine Rho-kinase (Amano *et al*., 1996a; Kimura *et al*., 1996). HA-tagged wild-type DRho-kinase was transiently expressed in 293 cells and an immune-complex kinase assay was performed. The wild-type DRho-kinase actively phosphorylated MLC and MBS (Fig. 3, lane 2) in addition to showing autophosphorylating activity (data not shown). On the contrary, DRho-kinase with Ala substituted for Lys116 at the ATP-binding site in the kinase domain showed no kinase activity (Fig. 3, lane 3), despite the fact that the mutant protein was expressed at a level comparable to the wild-type protein. The epitope-tagged mammalian Rho-kinase/ROKα proteins, which were expressed in cultured cells without co-transfection with *rhoA*, also demonstrated considerable kinase activities after immunoprecipitation (Matsui *et al*., 1996; Ishizaki *et al*., 1996; Leung *et al*., 1996). The kinase activity observed in this study suggests that a portion of the DRho-kinase was activated by human Rho proteins as previously suggested. Myelin basic protein (MBP) was also tested as a substrate. However, considerable levels of phosphorylation were detected even with the immunoprecipitates from the cells transfected with vector alone or cells expressing DRho-kinase^{K116A}, and only a marginal level of increase in phosphorylation was detected with the wild-type DRho-kinase (data not shown). The

result demonstrates that, like bovine Rho-kinase, DRho-kinase does phosphorylate MLC and MBS.

3. 4. Expression of DRho-kinase *during development*

To examine the tissue-specificity of *DRhk* expression in development, the distribution of the transcripts was analyzed by *in situ* hybridization with digoxigenin-labeled RNA probes. *DRhk* was expressed widely throughout embryogenesis (Fig. 4, A ~ F). The ubiquitous early expression of *DRhk* indicates a significant maternal contribution (Fig. 4A). In the imaginal discs from third instar larvae, the *DRhk* transcripts were detected at uniform levels (Fig. 4, G ~ L). The expression pattern was similar to the patterns observed for *Drho1* and its activator, *DRhoGEF2* (Hariharan *et al*., 1995; Barrett *et al*., 1997; Häcker and Perrimon, 1998).

3.5. Conclusion

We have identified a *Drosophila* homolog of Rho-kinase/ROKα, and its structural similarity to the vertebrate homologs suggests the functional conservation among them. In fact, DRho-kinase showed biochemical activities similar to those of mammalian Rho-kinase/ROKα; binding to Drho1 in GTP-form and a kinase activity toward MLC and MBS. MLC and MBS seem to be more preferable substrates for DRho-kinase than MBP. The *Drosophila* MLC encoded by *spaghetti-squash* (*sqh*) is strongly conserved, and the amino acid residues of chicken MLC around the site phosphorylated by MLC kinase are identical to those of *Drosophila* (Karess *et al*., 1991), suggesting that DRho-kinase phosphorylates the *Drosophila* MLC as well.

The DRho-kinase identified in this study is likely an effector of Drho1. Future studies on this gene may provide insights into the molecular mechanisms of morphogenetic events in development. Many of the components involved in the dynamic Rho-mediated cytoskeletal organization are conserved, so *Drosophila* would provide an excellent system for such studies.

Acknowledgement

We are grateful to J. Settleman for providing the cDNA clone. We are also greatly indebted to J. Ninomiya-Tsuji, K. Fujimura-Kamada and K. Matsumoto for technical advice and suggestions. We thank to T. Tsuboi and K. Dohmoto for technical assistance. This work was supported by grants from The Mitsubishi Foundation, the Ministry of Education, Science, Sports and Culture of Japan, and the Japan Science and Technology Corporation.

References

Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., Kaibuchi, K. 1996a. Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. Science 271, 648-650.

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., Kaibuchi, K. 1996b. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. 271, 20246-20249.

Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., Kaibuchi, K. 1997. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 275 1308-1311.

Barrett, K., Leptin, M., Settleman, J. 1997. The Rho GTPase and putative RhoGEF mediate a signaling pathway for the cell shape changes in *Drosophila* gastrulation. Cell 91, 905-915.

Bourne, H.R., Sanders, D.A., McCormick, F. 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature 349,117-127.

Crawford, J.M., Harden, N., Leung, T., Lim, L., Kiehart, D.P. 1998. Cellularization in *Drosophila melanogaster* is disrupted by the inhibition of Rho activity and the activation of Cdc42 function. Dev. Biol. 204, 151-164.

Eaton, S., Auvinen, P., Luo, L., Jan, Y.N., Simons, K. 1995. CDC42 and Rac1 control different actin-dependent processes in the *Drosophila* wing disc epithelium. J. Cell. Biol. 131, 151-164.

Farah, S., Agazie, Y., Ohan, N., Ngsee, J.K., Liu, X.J. 1998. A rho-associated protein kinase, ROKalpha, binds insulin receptor substrate-1 and modulates insulin signaling. J. Biol. Chem. 273, 4740-4746.

Fields, S., Song, O. 1989. A. novel genetic system to detect protein-protein interactions. Nature 340, 245-246.

Häcker, U., Perrimon, N. 1998. *DRhoGEF2* encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in *Drosophila*. Genes Dev. 12, 274-284.

Harden, N., Loh, H.Y., Chia, W., Lim, L. 1995. A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*. Development 121, 903-914.

Hariharan, I.K., Hu, K.Q., Asha, H., Quintanilla, A., Ezzell, R.M., Settleman, J. 1995. Characterization of rho GTPase family homologues in *Drosophila* melanogaster

overexpressing Rho1 in retinal cells causes a late developmental defect. EMBO J. 14, 292-302.

Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., Narumiya, S. 1996. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J. 15, 1885-1893.

Kaibuchi, K., Kuroda, S., Amano, M. 1999. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cell. Ann. Rev. Biochem. 68, 459-486.

Karess, R.E., Chang, X., Edwards, K.A., Kulkarni, S., Aguilera, I., Kiehart, D.P. 1991. The regulatory light chain of nonmuscle nmyosin is encoded by *spaghetti-squash*, a gene required for cytokinesis in *Drosophila*. Cell 65, 1177-1189.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., Kaibuchi, K. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273, 245-248.

Kozma, R., Ahmed, S., Best, A., Lim, L. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol. Cell. Biol. 15, 1942-1952.

Leung, T., Manser, E., Tan, L., Lim, L. 1995. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J. Biol. Chem. 270, 29051-29054.

Leung, T., Chen, X.Q., Manser, E., Lim, L. 1996. The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the

cytoskeleton. Mol. Cell. Biol. 16, 5313-5327.

Luo, L., Lee, T., Tsai, L., Tang, G., Jan, L.Y., Jan, Y.N. 1997. Genghis Khan (Gek) as a putative effector for *Drosophila* Cdc42 and regulator of actin polymerization. Proc. Natl. Acad. Sci. 94, 12963-12968.

Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A., Kaibuchi, K. 1996. Rho-associated kinase, as a putative target for small GTP binding protein Rho. EMBO J. 15, 2208-2216.

Murphy, A.M., Montell, D.J. 1996. Cell type-specific roles for Cdc42, Rac, and RhoL in Drosophila oogenesis. J. Cell. Biol. 133, 617-630.

Nakagawa, O., Fujisawa, K., Ishizaki, T., Saito, Y., Nakao, K., Narumiya, S. 1996. ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. FEBS Lett. 392, 189-193

Nikaido, M., Tada, M., Saji, T., Ueno, N. 1997. Conservation of BMP signaling in zebrafish mesoderm patterning. Mech. Dev. 61, 75-88.

Nobes, C.D., Hall, A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81, 53-62.

Ozaki, K., Tanaka, K., Imamura, H., Hihara, T., Kameyama, T., Nonaka, H., Hirano, H., Matsuura, Y., Takai, Y. 1996. Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. EMBO J. 15, 2196-2207.

Strutt, D.I., Weber, U., Mlodzik, M. 1997. The role of RhoA in tissue polarity and Frizzled signalling. Nature 387, 292--295.

Umemiya, T., Takeich,i M., Nose, A. 1997. M-spondin, a novel ECM protein highly

homologous to vertebrate F-spondin, is localized at the muscle attachment sites in the *Drosophila* embryo. Dev. Biol. 186, 165-176.

Vojtek, A.B., Hollenberg, S.M., Cooper, J.A. 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 74, 205-214.

Wissmann, A., Ingles, J., McGhee, J.D., Mains, P.E. 1997. *Caenorhabditis elegans* LET-502 is related to Rho-binding kinases and human myotonic dystrophy kinase and interacts genetically with a homolog of the regulatory subunit of smooth muscle myosin phosphatase to affect cell shape. Genes. Dev. 11, 409-422.

Figure legends

Fig. 1. Primary structure of DRho-kinase and a comparison with its homologs. (A) The predicted protein sequence of DRho-kinase. The sequence in the kinase domain is underlined. (B) Schematic representation of the basic structural features of the Rho-kinase/ROKα family proteins. The N-terminal kinase domains are indicated with filled boxes and the central coiled-coil, and the C-terminal PH domains are shaded or hatched, respectively. The percentages of the amino acids identical to the amino acid residues in the catalytic domain of DRho-kinase are indicated. The putative Rho-binding sites are underlined. (C) Alignment of amino acid sequences in the kinase domain. The amino acid sequence in the catalytic domain of DRho-kinase is aligned with that of bovine Rho-kinase (Amano *et al*. 1996a), rat ROKα (Leung *et al*., 1996), *Xenopus* ROKα (Farah *et al*., 1998), and *C. elegans* LET-502 (Wissmann *et al*., 1997). Identical residues are indicated with reverse type. The sequences used for the degenerate PCR primers are indicated with lines over the *Drosophila* sequence. The asterisk indicates

Lys116, the ATP-binding site, which was substituted with Ala in DRho-kinase^{K116A}.

(D) Alignment of amino acid sequences in the putative Rho-binding site. The *Drosophila* sequence is compared as above. The asterisks indicate the conserved adjacent Asn and Lys residues which have been demonstrated to be essential for Rho-binding in rat ROKα (Leung *et al*., 1996).

Fig. 2. Two hybrid analyses of the interaction of DRho-kinase with Drho1. (A) Full-length DRho-kinase interacts with GTP-bound Drho1. The reporter strain L40 was transfomed with the pBTM116 and pACTII vectors with indicated inserts. Lines indicate no insert. The transformants were plated on a synthetic medium lacking histidine and containing 10 mM 3-amino triazole and incubated at 30°C for 48 to 72 hr. Three independent transformants are shown. (B) The fragment of DRho-kinase, spanning from residue 912 to 1107, interacts with Drho1 in GTP form. L40 was transformed with the pBTM116 and pACTII vectors with indicated inserts and incubated as above. The conserved Asn and Lys residues were replaced with Thr residues in DRho-kinae(912-1077)TT.

Fig. 3. Protein kinase activity of DRho-kinase. HA-tagged DRho-kinase expressed in 293 cells was immunoprecipitated and the precipitates were used for *in vitro* kinase assay with MBS or MLC as substrates. Lane 1; vector alone, lane 2; wild-type DRho-kinase, and lane 3; DRho-kinase^{K116A}. Portions of the immunoprecipitates were tested for the levels of expression by immunoblotting.

Fig. 4. Expression of *DRho-kinase* during development. Anti-sense (A, C, E, G, I and K) or sense RNA (B, D, F, H, J and L) probes were used to detect the transcripts. (A to F) Expression during embryogenesis. Blastoderm embryos (A and B), germband-elongated embryos (C and D) and germband-retracted embryos (E and F) are shown. Anterior is to the left and dorsal to the top. (G to L) Expression in the imaginal discs of third instar larvae. Eye-antennal discs (G and H), wing discs (I and H) and leg discs (K and L) are shown.

Fig. 1.

A

```
   1
MPAGRETVTKQRSMDVERRRRANTLEREMRDPTSICNVDCLLDTVSALVSDCDHESLRRL
  61
KNIEQYAAKYKPLAMQINQLRMNVEDFHFIKLIGAGAFGEVQLVRHKSSSQVYAMKRLSK
 121
FEMMKRPDSAFFWEERHIMAHANSEWIVQLHFAFQDAKYLYMVMDFMPGGDIVSLMGDYD
 181
IPEKWAIFYTMEVVLALDTIHNMGFVHRDVKPDNMLLDSYGHLKLADFGTCMRMGANGQV
 241
VSSNAVGTPDYISPEVLQSQGVDNEYGRECDWWSVGIFLYEMLFGETPFYADSLVGTYGK
 301
IMDHKNSLSFPPEVEISEQAKALIRAFLTDRTQRLGRYGIEDIKAHPFFRNDTWSFDNIR
 361
ESVPPVVPELSSDDDTRNFEDIERDEKPEEVFPVPKGFDGNHLPFIGFTYTGDYQLLSSD
 421
TVDAESKEANVANSGAASNNHGHGHNHRHRPSNSNELKRLEALLERERGRSEALEQQDAG
 481
LRQQIELITKREAELQRIASEYEKDLALRQHNYKVAMQKVEQEIELRKKTEALLVETQRN
 541
LENEQKTRARDLNINDKVVSLEKQLLEMEQSYKTETENTQKLKKHNAELDFTVKSQEEKV
 601
RDMVDMIDTLQKHKEELGQENAELQALVVQEKNLRSQLKEMHKEAENKMQTLINDIERTM
 661
CREQKAQEDNRALLEKISDLEKAHAGLDFELKAAQGRYQQEVKAHQETEKSRLVSREEAN
```

B

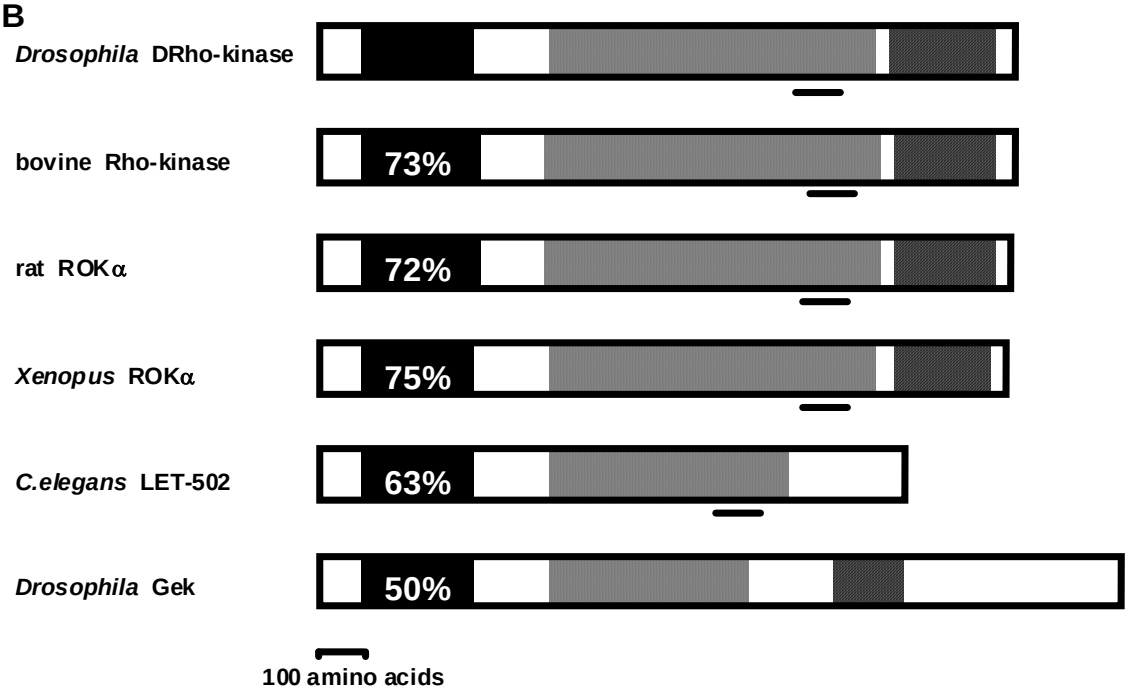


Fig. 2.

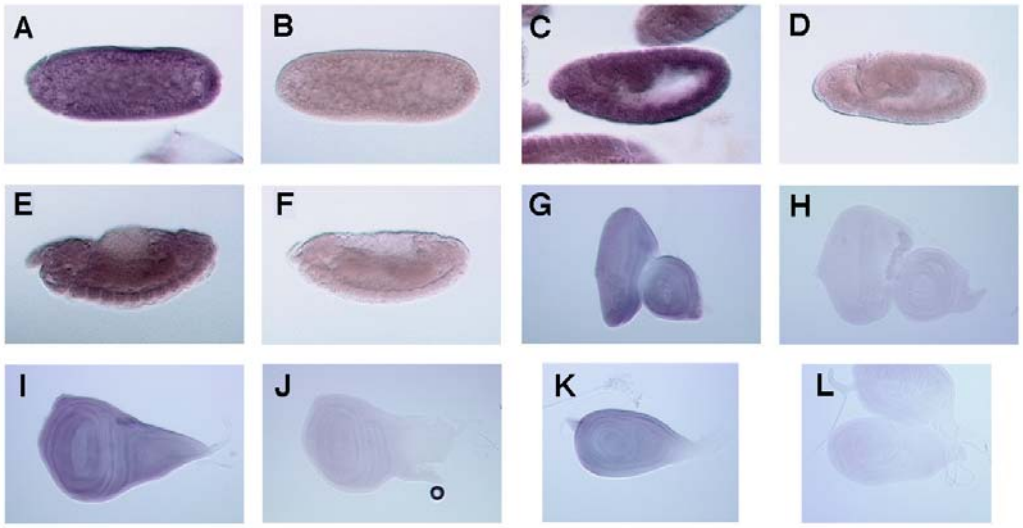


Fig. 3.

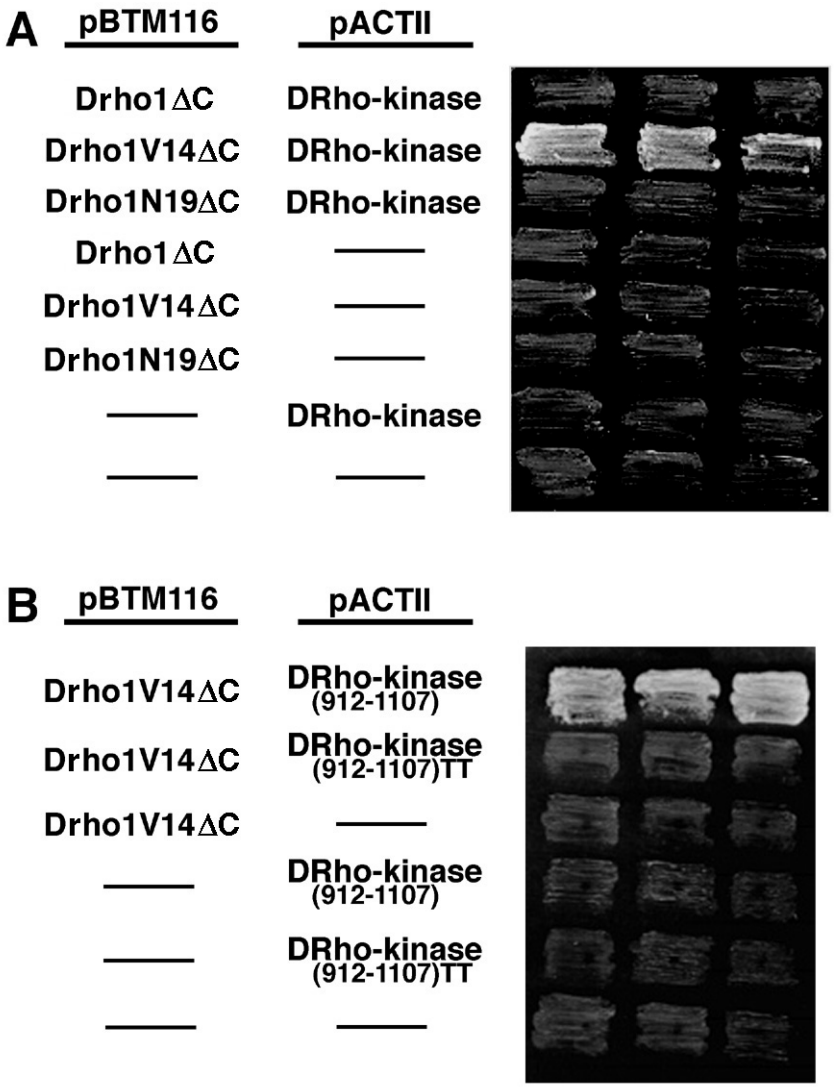
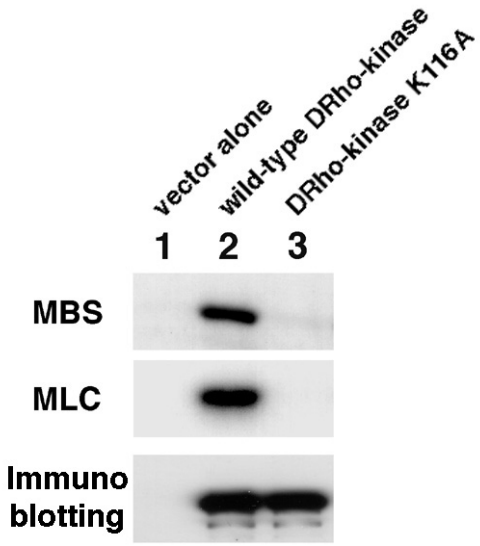

A
pBTM116
pACTII
Drho1ΔC
DRho-kinase
Drho1V14ΔC
DRho-kinase
Drho1N19ΔC
DRho-kinase
Drho1ΔC
Drho1V14ΔC
Drho1N19ΔC
DRho-kinase
B
pBTM116
pACTII
Drho1V14ΔC
DRho-kinase (912-1107)
Drho1V14ΔC
DRho-kinase (912-1107)TT
Drho1V14ΔC
DRho-kinase (912-1107)
DRho-kinase (912-1107)TT

Fig. 4.


vector alone
wild-type DRho-kinase
DRho-kinase K116A
1
2
3
MBS
MLC
Immuno blotting