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Rho-associated kinase, a novel serine/threonine kinase, as a putative target for the small GTP binding protein Rho

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The small GTP binding protein Rho is implicated in cytoskeletal responses to extracellular signals such as lysophosphatidic acid to form stress fibers and focal contacts. Here we have purified a Rho-interacting protein with a molecular mass of ~164 kDa (p164) from bovine brain. This protein bound to GTPγS (a non-hydrolyzable GTP analog)·RhoA but not to GDP·RhoA or GTPγS·RhoA with a mutation in the effector domain ($RhoA^{A37}$). p164 had a kinase activity which was specifically stimulated by GTPγS·RhoA. We obtained the cDNA encoding p164 on the basis of its partial amino acid sequences and named it Rho-associated kinase (Rho-kinase). Rho-kinase has a catalytic domain in the N-terminal portion, a coiled coil domain in the middle portion and a zinc finger-like motif in the C-terminal portion. The catalytic domain shares 72% sequence homology with that of myotonic dystrophy kinase and the coiled coil domain contains a Rho-interacting interface. When COS7 cells were co-transfected with Rho-kinase and activated RhoA, some Rho-kinase was recruited to membranes. Thus it is likely that Rho-kinase is a putative target serine/threonine kinase for Rho and serves as a mediator of the Rho-dependent signaling pathway.

Keywords: cytoskeleton/GTP binding protein/phosphorylation/protein kinase/Rho

Introduction

The Rho family, like other GTP binding proteins, exhibits both GDP/GTP binding and GTPase activities (Nobes and Hall, 1994). They have GDP-bound inactive and GTP-bound active forms, which are interconvertible by GDP/GTP exchange and GTPase reactions (Nobes and Hall, 1994). The GDP/GTP exchange reaction is regulated by stimulatory proteins such as Smg GDS (Kaibuchi *et al.*, 1991; Mizuno *et al.*, 1991), Dbl (Hart *et al.*, 1991), Ost (Horii *et al.*, 1994) and Tiam-1 (Habets *et al.*, 1994) and by inhibitory proteins such as Rho GDI (Fukumoto *et al.*, 1990). The GTPase reaction is regulated by Rho GTPase-activating proteins (GAP) such as Ras GAP-associated p190 (Settleman *et al.*, 1992), Rho GAP (Lancaster *et al.*, 1994) and Rho GAP p122 (Homma and Emori, 1995). Members of the Rho family of proteins, including RhoA, RhoB, RhoC, Rac1, Rac2 and Cdc42 share >50% sequence identity (Nobes and Hall, 1994). Rho is implicated in the appropriate responses of the cytoskeletal network to extracellular signals such as lysophosphatidic acid and certain growth factors to form stress fibers and focal contacts (Ridley and Hall, 1992, 1994). Rho is also implicated in other physiological functions associated with cytoskeletal rearrangements, such as cell morphology (Paterson *et al.*, 1990), aggregation (Tominaga *et al.*, 1993), motility (Takaishi *et al.*, 1994) and cytokinesis (Kishi *et al.*, 1993; Mabuchi *et al.*, 1993). Recent studies indicate that Rho is also involved in regulating smooth muscle contraction (Hirata *et al.*, 1992), phosphatidylinositol 3-kinase (PI 3-kinase) (Kumagai *et al.*, 1993; Zhang *et al.*, 1993), phosphatidylinositol 4-phosphate 5-kinase (PI 4,5-kinase) (Chong *et al.*, 1994) and c-*fos* expression (Hill *et al.*, 1995). Rac is involved in the control of superoxide generation by NADPH oxidase in neutrophils (Abo *et al.*, 1991; Knaus *et al.*, 1991; Mizuno *et al.*, 1992). Activated Rac assembles with membrane-bound cytochrome b to form an active NADPH oxidase together with p47-*phox* and p67-*phox* (Abo *et al.*, 1991; Knaus *et al.*, 1991; Mizuno *et al.*, 1992). Activated Rac induces the formation of membrane ruffling and lamellipodia in fibroblasts (Ridley *et al.*, 1992). Cdc42 was originally identified in the yeast *Saccharomyces cerevisiae*, where it regulates polarized cell growth (Johnson and Pringle, 1990). Cdc42 regulates the formation of actin-containing microspikes, called filopodia, in fibroblasts (Kozma *et al.*, 1995; Nobes and Hall, 1995).

Upon stimulation with certain extracellular signals, the GDP-bound form of the Rho family of proteins may be converted to the GTP-bound form, which bind to specific targets and exert their biological functions (Nobes and Hall, 1994). Cdc42 appears to be downstream of bradykinin, Rac appears to be downstream of PDGF and insulin and Rho appear to be downstream of lysophosphatidic acid (Kozma *et al.*, 1995; Nobes and Hall, 1995). In Swiss 3T3 cells, a cascade of Cdc42 controlling Rac which controls Rho may coordinate the actin cytoskeleton during cell movement (Kozma *et al.*, 1995; Nobes and Hall, 1995). For example, bradykinin appears to stimulate the cascade via Cdc42, producing the effect of Cdc42 activation (filopodia) followed by Rac activation (lamellipodia) and subsequent Rho activation (stress fibers and focal contacts) (Kozma *et al.*, 1995; Nobes and Hall, 1995).

The target for the GTP-bound active form of Rac has been identified as p67-*phox* in the NADPH oxidase system (Diekmann *et al.*, 1994). An additional target for Rac and Cdc42 is serine/threonine kinase PAKp65, which is

activated by Rac and Cdc42 (Manser *et al.*, 1994; Martin *et al.*, 1995). The p85 subunit of PI 3-kinase is also directly associated with activated Rac and Cdc42 (Zheng *et al.*, 1994). However, specific targets for Rho have not yet been identified.

Here we have investigated the putative targets for Rho in order to understand its mode of action. We purified one target, characterized its biochemical properties, cloned its cDNA, identified it as a novel serine/threonine kinase and named it Rho-associated kinase (Rho-kinase).

Results

Purification of Rho-interacting protein p164

We have recently identified three species of Rho-interacting proteins with molecular masses of ~128, 164 and 180 kDa (p128, p164 and p180 respectively) by use of GST–Rho affinity column chromatography (Amano *et al.*, 1996). A membrane extract of bovine brain was loaded onto a glutathione–Sepharose affinity column on which glutathione *S*-transferase (GST), GDP·GST–RhoA or GTPγS (a non-hydrolyzable GTP analog)·GST–RhoA was immobilized. The proteins bound to the affinity columns were then co-eluted with GST–RhoA by addition of glutathione. This procedure yielded p128, p164 and p180 in the eluate from the GTPγS·GST–RhoA, but not from the GST or GDP·GST–RhoA, affinity columns (Figure 1A). These proteins were scarcely retained on the GTPγS·GST–RhoA^{A37} (which contains an amino acid substitution in the effector domain) affinity column (data not shown). Neither p128, p164 nor p180 were eluted from GTPγS·GST–Rac1 or GTPγS·GST–H-Ras affinity columns (data not shown). Thus it is most likely that p128, p164 and p180 interact specifically with activated RhoA via the effector domain. Among the identified RhoA-interacting proteins we enriched p164 by means of specific elution from the GTPγS·GST–RhoA affinity column in the presence of 1% 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid (CHAPS) (Figure 1A). The partially purified p164 was further purified by Mono Q column chromatography. p164 protein eluted as a single peak in the column chromatography and was purified to near homogeneity (>95%) (Figure 1B).

Direct binding of GTPγS·RhoA to p164

The overlay assay for small GTP binding proteins was initially described as a method of detecting GAP and small GTP binding protein-interacting proteins (Manser *et al.*, 1992). By probing a nitrocellulose filter containing the crude proteins with radioactive small GTP binding proteins, those that interact with small GTP binding proteins can be detected (Manser *et al.*, 1992). To examine the direct binding of GTPγS·GST–RhoA to p164 an overlay assay with [^{35}S]GTPγS·GST–RhoA was carried out. [^{35}S]GTPγS·GST–RhoA bound to p164 in the membrane extract and purified preparation, but [^{35}S]GTPγS·GST–RhoA^{A37} did not (Figure 2), indicating that activated RhoA binds directly to p164 via the effector domain. [^{35}S]GTPγS·GST–Rac1 did not bind to p164 (data not shown). [γ-^{32}P]GTP·GST–RhoA has been shown to bind a protein with a M_r of ~145 kDa (p145), which may correspond to p164 (Manser *et al.*, 1994).

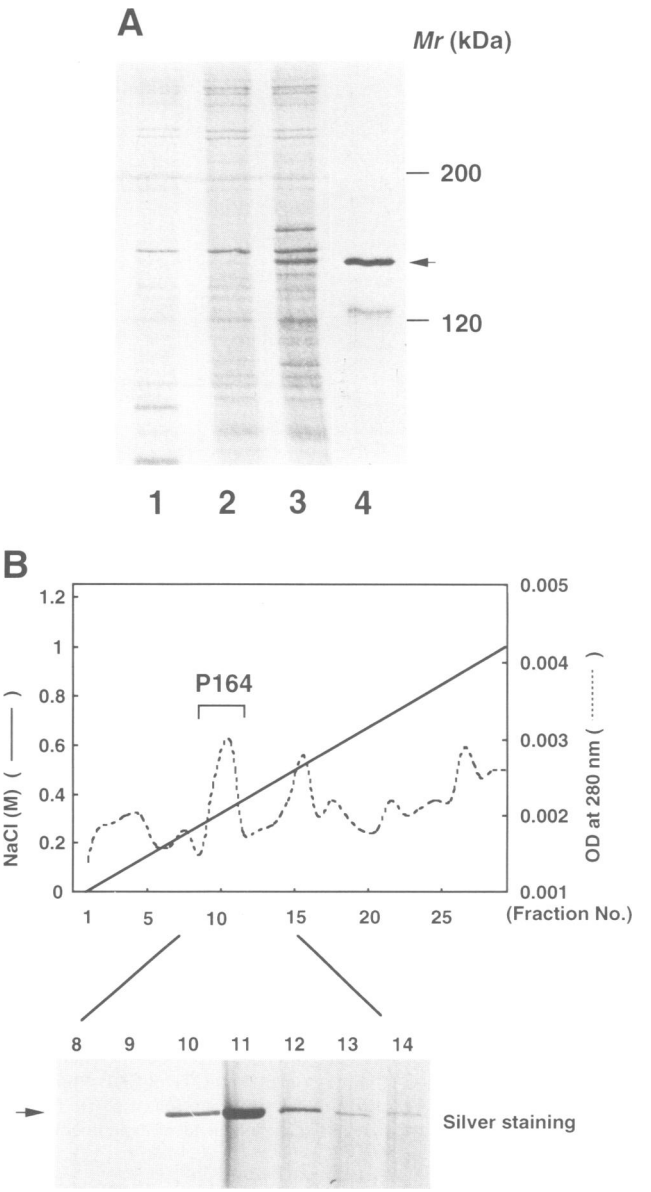


Fig. 1. (**A**) Purification of Rho-interacting proteins. The crude membrane fraction was loaded onto a glutathione–Sepharose column containing either GST (lane 1), GDP·GST–RhoA (lane 2) or GTPγS·GST–RhoA (lane 3). The bound proteins were co-eluted with the respective GST fusion proteins by addition of glutathione. To enrich p164 the crude membrane fraction was loaded onto a glutathione–Sepharose column containing GTPγS·GST–RhoA and p164 protein was eluted by addition of 1% CHAPS (lane 4). Aliquots of the eluates were resolved by SDS–PAGE followed by silver staining. (**B**) Purification of p164 by Mono Q column chromatography. The CHAPS eluate was subjected to a Mono Q column and p164 was eluted with a linear gradient of NaCl. The arrow denotes the position of p164. The results shown are representative of three independent experiments.

p164 as a protein kinase

We have recently identified the Rho-interacting protein p128 as protein kinase N (PKN) (Mukai and Ono, 1994; Amano *et al.*, 1996). This led us to examine whether p164 shows kinase activity. Purified p164 exhibited autophosphorylation *in vitro* in the presence of [γ-^{32}P]ATP (Figure 3). We tested whether GTP·RhoA modulates the kinase activity of p164. When p164 underwent autophosphoryl-

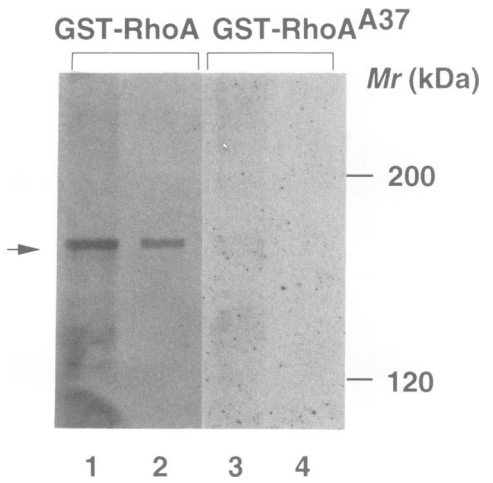


Fig. 2. Direct binding of activated RhoA to p164. Nitrocellulose membranes containing the membrane extract (lanes 1 and 3) and purified p164 (lanes 2 and 4) separated by SDS–PAGE were probed with [^{35}S]GTPγS·GST–RhoA (lanes 1 and 2) or [^{35}S]GTPγS·GST–RhoA^{A37} (lanes 3 and 4). The arrow denotes the position of p164. The results shown are representative of three independent experiments.

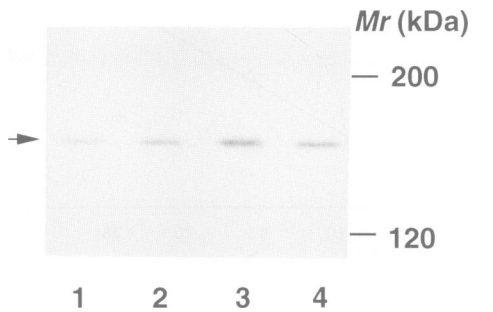
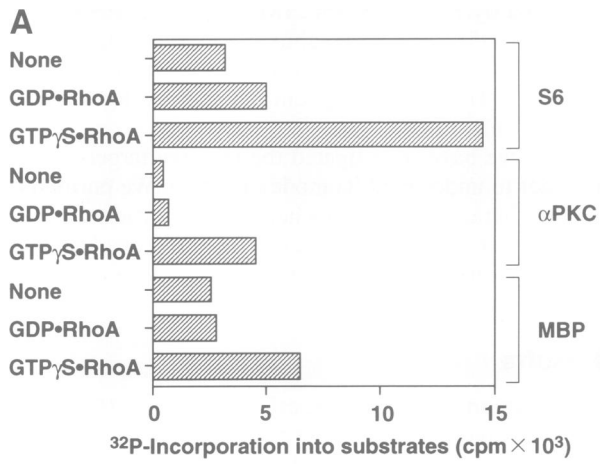


Fig. 3. Autophosphorylation of p164. The p164 protein was autophosphorylated in the presence of either GST (lane 1), GDP·GST–RhoA (lane 2), GTPγS·GST–RhoA (lane 3) or GTPγS·GST–RhoA^{A37} (lane 4) (1 μM each). The arrow denotes the position of p164. The results shown are representative of three independent experiments.

ation we found that GTPγS·GST–RhoA stimulated the reaction up to 2-fold. GDP·GST–RhoA and GTPγS·GST–RhoA^{A37} were less effective. We then examined the kinase activity of p164 toward exogenous substrates, such as S6 peptide (RRRLSSLRA). p164 phosphorylated S6 peptide and GTPγS·GST–RhoA stimulated this reaction, whereas GDP·GST–RhoA had a much weaker effect (Figure 4A). The effect of GTPγS·GST–RhoA was dose dependent and the EC_{50} for GTPγS·GST–RhoA was ~600 nM. We also examined the phosphorylation of a serine-containing synthetic peptide based on the protein kinase Cα (PKCα) pseudosubstrate (αPKC peptide) (RFARKGSLRQKNVHEVK) and myelin basic protein and found that GTPγS·GST–RhoA stimulated kinase activity toward PKCα peptide and myelin basic protein (Figure 4A). Among these substrates, S6 peptide was the preferred substrate for p164. We next examined the effect of various small GTP binding proteins on the kinase activity of p164 and found that GTPγS·GST–RhoA^{A37} and GTPγS·GST-Rac1 had only a residual effect and GTPγS·GST-H-Ras had no effect (Figure 4B). Since Rho is implicated in cytoskeletal rearrangements, we searched for p164 substrates among cytoskeletal regulatory proteins, including vinculin, talin, metavinculin, caldesmon, filamin, vimentin, α-actinin (Clark and Brugge, 1995), MAP-4 (Aizawa *et al.*, 1990) and the myosin binding subunit of myosin phosphatase (Chen *et al.*, 1994; Shimizu *et al.*, 1994a). Among these the myosin binding subunit was the preferred substrate for p164. GTPγS·GST–RhoA stimulated kinase activity toward the myosin binding subunit ~15-fold (Figure 5). (A detailed analysis concerning phosphorylation of the myosin binding subunit will be described elsewhere.)



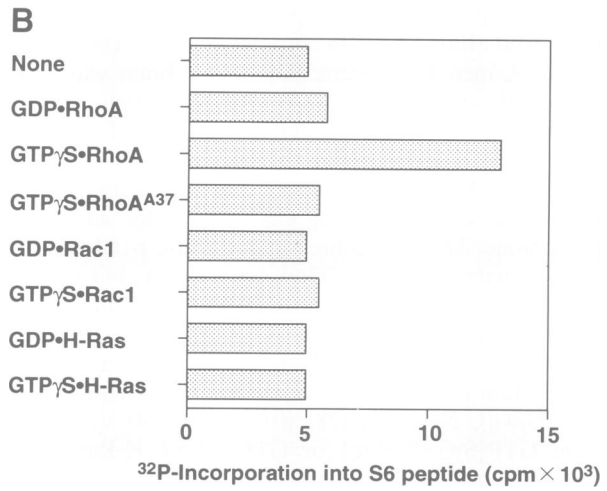


Fig. 4. Phosphorylation of exogenous substrates by p164. (**A**) Phosphorylation of various substrates by p164. The kinase reaction was carried out with S6 peptide, αPKC peptide or myelin basic protein (MBS) (40 μM each) in the presence of either GST, GDP·GST–RhoA or GTPγS·GST–RhoA (1 μM each) as indicated. (**B**) Effects of various small G proteins on the kinase activity of p164. The kinase reaction was carried out with S6 peptide (40 μM) in the presence of various GST-immobilized small G proteins (1 μM each) as indicated. The results shown are representative of three independent experiments.

Isolation of the p164 cDNA

To clarify the molecular identity of p164, purified protein was subjected to amino acid sequencing as described in Materials and methods. Thirty-seven peptide sequences derived from p164 were determined. Two sequences of the peptides were used to design degenerate oligonucleotide probes for cDNA cloning. By use of degenerate oligonucleotide probes, cDNA encoding the complete sequence of p164 was isolated from a bovine cDNA library. The

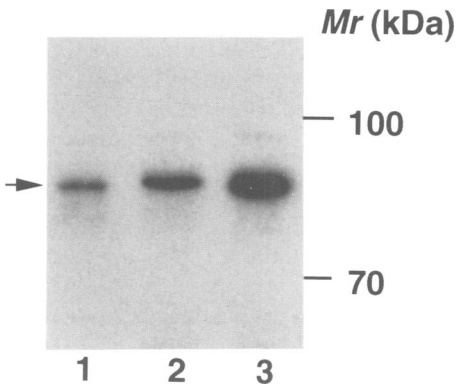


Fig. 5. Phosphorylation of myosin binding subunit by p164. The kinase reaction was carried out with the C-terminus of rat myosin binding subunit (amino acids 699–976) as a fusion protein with maltose binding protein (50 nM) in the presence of either GST (lane 1), GDP·GST–RhoA (lane 2) or GTPγS·GST–RhoA (lane 3) (1 μM each). The arrow denotes the position of the myosin binding subunit. The results shown are representative of three independent experiments.

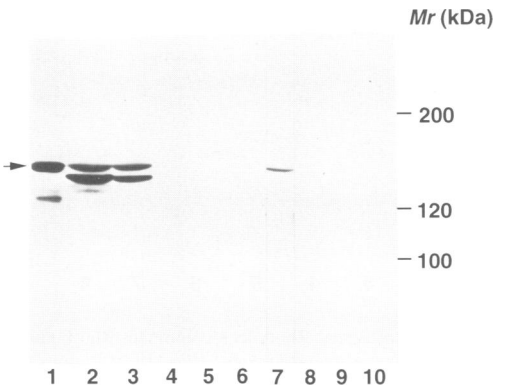


Fig. 7. Immunoblot analysis of Rho-kinase. Immunoblot analysis of Rho-kinase was carried out using an anti-Rho-kinase antibody (against CKRQLQERFTDLEK). Lane 1, 1% CHAPS eluate from the GST–RhoA affinity column; lane 2, cerebrum; lane 3, cerebellum; lane 4, heart; lane 5, skeletal muscle; lane 6, spleen; lane 7, lung; lane 8, liver; lane 9, kidney; lane 10, pancreas. The arrow denotes the position of Rho-kinase. The results shown are representative of three independent experiments.

A

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MSRPPPTGKM PGAPEAVSGD GAGASRQRKL EALIRDPRSP INVESLLDGL NPLVLDLDFP   60
ALRKNKNIDN FLNRYEKIVK KIRGLQMKAE DYDVVKVIGR GAFGEVQLVR HKASQKVYAM  120
KLLSKFEMIK RSDSAFFWEE RDIMAFANSP WVVQLFCAFQ DDKYLYMVME YMPGGDLVNL  180
MSNYDVPEKW AKFYTAEVVL ALDAIHSMGL IHRDVKPDNM LLDKHGHLKL ADFGTCMKMD  240
ETGMVHCDTA VGTPDYISPE VLKSQGGDGY YGRECDWWSV GVFLFEMLVG DTPFYADSLV  300
GTYSKIMDHK NSLCFPEDAE ISKHAKNLIC AFLTDREVRL GRNGVEEIKQ HPFFKNDQWN  360
WDNIRETAAP VVPELSSDID SSNFDDIEDD KGDVETFPIP KAFVGNQLPF IGFTYYRENL  420
LLSDSPSCKE NDSIQSRKNE ESQEIQKKLY TLEEHLSTEI QAKEELEQKC KSVNTRLEKV  480
AKELEEEITL RKNVESTLRQ LEREKALLQH KNAEYQRKAD HEADKKRNLE NDVNSLKDQL  540
EDLKKRNQNS QISTEKVNQL QRQLDETNAL LRTESDTAAR LRKTQAESSK QIQQLESNNR  600
DLQDKNCLLE TAKLKLEKEF INLQSVLESE RRDRTHGSEI INDLQGRISG LEEDVKNGKI  660
LLAKVELEKR QLQERFTDLE KEKNNMEIDM TYQLKVIQQS LEQEETEHKA TKARLADKNK  720
IYESIEEAKS EAMKEMEKKL SEERTLKQKV ENLLLEAEKR CSILDCDLKQ SQQKINELLK  780
QKDVLNEDVR NLTLKIEQET QKRCLTQNDL KMQTQQVNTL KMSEKQLKQE NNHLLEMKMS  840
LEKQNAELRK ERQDADGQMK ELQDQLEAEQ YFSTLYKTQV RELKEECEEK TKLCKELQQK  900
KQELQDERDS LAAQLEITLT KADSEQLARS IAEEQYSDLE KEKIMKELEI KEMMARHKQE  960
LTEKDATIAS LEETNRTLTS DVANLANEKE ELNNKLKEAQ EQLSRLKDEE ISAAAIKAQF 1020
EKQLLTERTL KTQAVNKLAE IMNRKEPVKR GNDTDVRRKE KENRKLHMEL KSEREKLTQQ 1080
MIKYQKELNE MQAQIAEESQ IRIELQMTLD SKDSDIEQLR SQLQALHIGL DSSSIGSGPG 1140
DTEADDGFPE SRLEGWLSLP VRNNTKKFGW VKKYVIVSSK KILFYDSEQD KEQSNPYMVL 1200
DIDKLFHVRP VTQTDVYRAD AKEIPRIFQI LYANEGESKK EQEFPVEPVG EKSNYICHKG 1260
HEFIPTLYHF PTNCEACMKP LWHMFKPPPA LECRRCHIKC HKDHMDKKEE IIAPCKVYYD 1320
ISSAKNLLLL ANSTEEQQKW VSRLVKKIPK KPPAPDPFAR SSPRTSMKIQ QNQSIRRPSR 1380
QLAPNKPS                                                          1388
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B

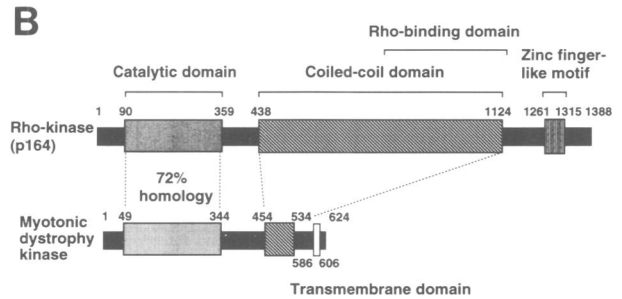


Fig. 6. Structure of Rho-kinase. (**A**) Deduced amino acid sequence of Rho-kinase. Those sequences determined from native Rho-kinase are indicated by single underlining. Amino acid sequences used for oligonucleotide probes are indicated by double underlining. The GenBank accession no. is U36909. (**B**) Schematic representation of Rho-kinase and myotonic dystrophy kinase.

predicted protein contains 1388 amino acid residues, with a calculated molecular mass of 160.797 kDa, which is close to its apparent M_r of 164 kDa estimated by SDS–PAGE (Figure 2). The deduced amino acid sequence is shown in Figure 6A. All 37 peptide sequences obtained were found within the deduced amino acid sequence. The neighboring sequence around the initiation codon was consistent with the translation initiation start site proposed by Kozak (1987) and we found an in-frame termination codon in the preceding region. The predicted protein product has a putative kinase domain in the N-terminal portion and it shares 72% sequence homology with myotonic dystrophy kinase within the kinase domain (Figure 6B) (Brook *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*, 1992). The p164 protein has a putative coiled coil structure in the middle portion, which shows a significant similarity with the myosin rod, and a zinc finger-like motif in the C-terminal portion. Based on these results we designated p164 as Rho-associated kinase (Rho-kinase).

Tissue distribution of Rho-kinase

We examined the expression of Rho-kinase in several rat tissues by immunoblot analysis. We generated antibodies against two peptides derived from the amino acid sequence of bovine Rho-kinase. These antibodies cross-reacted with rat Rho-kinase. Immunoblot analysis was carried out by use of one of the antibodies to detect Rho-kinase in various rat tissues. Rho-kinase was expressed at high levels in the cerebrum and cerebellum, weakly in the heart and lung and hardly in skeletal muscle, the spleen, liver, kidney and pancreas (Figure 7). The faster migrating bands in the cerebrum and cerebellum may be degraded p164 or an isoform. Essentially identical results were obtained when the other antibody was employed. These results suggest that Rho-kinase is expressed at high levels in the rat brain. By overlay assay with [^{35}S]GTPγS·RhoA as a probe bands corresponding to Rho-kinase were detected in various rat tissues, including the brain, heart, kidney, liver, lung, spleen and testis (data not shown; Manser *et al.*, 1994). In preliminary experiments, we also identified a similar kinase activity in the chicken gizzard. Thus it is likely that Rho-kinase-like molecules are expressed in various tissues.

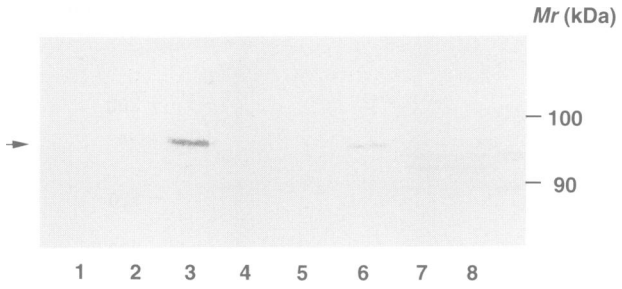


Fig. 8. Binding of activated RhoA to recombinant Rho-kinase. The *in vitro* translated coiled coil domain of Rho-kinase (amino acids 421–1137) was mixed with GST-immobilized small G proteins attached to glutathione–Sepharose 4B beads. The interacting proteins were eluted with GST-immobilized small G proteins by addition of glutathione. Aliquots were resolved by SDS–PAGE followed by autoradiography. Lane 1, GST; lane 2, GDP·GST–RhoA; lane 3, GTPγS·GST–RhoA; lane 4, GTPγS·GST–RhoA^{A37}; lane 5, GDP·GST–Rac1; lane 6, GTPγS·GST–Rac1; lane 7, GDP·GST–H-Ras; lane 8, GTPγS·GST–H-Ras. The arrow denotes the position of the *in vitro* translated Rho-kinase. The results shown are representative of three independent experiments.

Complex formation between recombinant Rho-kinase and RhoA

To address whether recombinant Rho-kinase interacts with GTPγS·RhoA, GST-immobilized small G proteins were mixed with the *in vitro* translated coiled coil domain of Rho-kinase (amino acids 421–1137) and interacting proteins were eluted with GST-immobilized small G proteins by addition of glutathione. The *in vitro* translated coiled coil domain was retained on the GTPγS·GST–RhoA affinity beads and was co-eluted with GTPγS·GST–RhoA by addition of glutathione, whereas it was retained only slightly on the GTPγS·GST–Rac1 and not at all on the GST, GDP·GST–RhoA, GTPγS·GST–RhoA^{A37} or GTPγS·GST–H-Ras affinity beads (Figure 8). Essentially identical patterns of retension were observed when the shorter coiled coil domain of Rho-kinase (amino acids 799–1137) was employed (data not shown). These results indicate that GTPγS·GST–RhoA directly interacts with the C-terminal portion of the coiled coil domain (Figure 6B).

Intracellular localization of Rho-kinase

By use of an overlay assay with radioactive RhoA as a probe, we measured the amount of Rho-kinase in bovine brain and found that ~25% of Rho-kinase was present in the particulate fraction and ~75% was present in the cytosol fraction.

We examined the intracellular localization of Rho-kinase in COS7 cells. When COS7 cells were transfected with myc epitope-tagged Rho-kinase (myc–Rho-kinase) ~96% myc–Rho-kinase was present in the cytosol fraction and ~4% myc–Rho-kinase was present in the particulate fraction (Figure 9A). There was an increase in myc–Rho-kinase in the particulate fraction when the cells were co-transfected with hemagglutinin (HA)-epitope tagged RhoA (HA–RhoA). Activated HA–RhoA (HA–RhoA^{V14}) was more effective than HA–RhoA in this capacity. About 50% each of HA–RhoA and HA–RhoA^{V14} were present in the particulate fraction. These results suggest that HA–RhoA promotes the association of myc–Rho-kinase with membranes, presumably by forming a complex with myc–Rho-kinase.

For immunofluorescence analysis, we transfected myc–Rho-kinase into NIH 3T3 cells, examined the localization of myc–Rho-kinase by use of an anti-myc antibody and found diffuse immunofluorescence staining throughout the cytoplasm, most likely representing cytosolic protein (Figure 9B). myc–Rho-kinase did not apper to be co-localized with actin filaments. This observation is in agreement with the subcellular fractionation experiments. Co-transfection of HA–RhoA^{V14} did not appear to promote translocation of myc–Rho-kinase to specific sites (data not shown). This may be due to the low sensitivity of this method.

Discussion

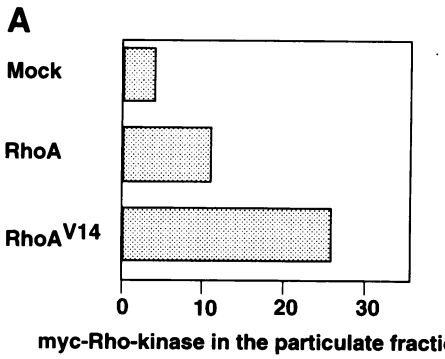
Here we have purified a protein that specifically interacts with activated RhoA by means of GST–RhoA affinity column chromatography. We have cloned its cDNA, identified it as a novel serine/threonine kinase and named it Rho-kinase. We have shown that activated RhoA interacts directly with Rho-kinase and activates it in a cell-free system. Furthermore, we have shown that overexpression of activated RhoA promotes association of Rho-kinase with membranes in COS7 cell. Thus it is likely that Rho-kinase is a putative target serine/threonine kinase for Rho and serves as a mediator of the Rho-dependent signaling pathway.

Interface between activated Rho and its targets

We have shown that RhoA interacts directly with the C-terminal portion of the coiled coil domain of Rho-kinase, indicating that this unique domain confers specificity for the GTP·Rho complex. We have recently found that Rho-interacting p128 is PKN and that PKN may also serve as a putative target for Rho (Amano *et al.*, 1996). PKN is a serine/threonine kinase composed of a N-terminal regulatory domain and a C-terminal catalytic domain (Mukai and Ono, 1994). The interface of the GTP·Rho interaction has been localized to the N-terminal regulatory domain of PKN, which contains a polybasic region followed by a leucine zipper-like motif. There is no obvious sequence homology between the coiled coil domain of Rho-kinase and the N-terminal regulatory domain of PKN, suggesting that activated Rho can recognize at least two different types of target interfaces.

Mode of activation of Rho-kinase by Rho

The mode of activation of PAKp65 has been characterized (Manser *et al.*, 1994; Martin *et al.*, 1995). GTPγS·Rac1 or GTPγS·Cdc42 dramatically stimulate autophosphorylation of PAKp65. Autophosphorylation induced by Rac1 or Cdc42 stimulates the kinase activity of PAKp65 toward exogenous substrates, such as myelin basic protein. Once PAKp65 is activated, Rac1 or Cdc42 is no longer required to keep it activated. On the other hand, GTPγS·RhoA stimulates autophosphorylation of Rho-kinase up to 2-fold under conditions where it stimulates Rho-kinase activity toward the myosin binding subunit >15-fold. Thus it is unlikely that GTP·RhoA stimulates Rho-kinase activity through autophosphorylation. We have shown that stimulation of Rho-kinase activity by RhoA depends on the substrate. We assume that activated RhoA directly interacts with the C-terminal portion of the coiled coil domain of



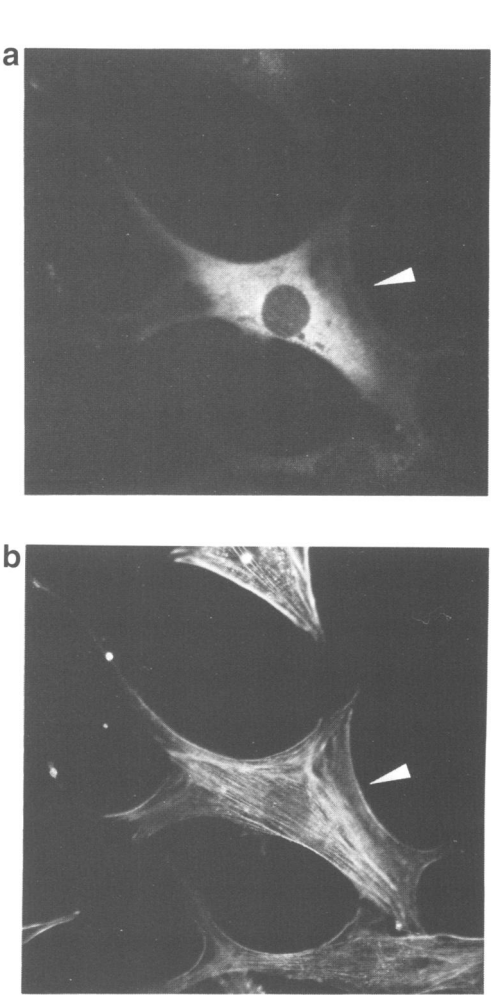


Fig. 9. Intracellular localization of Rho-kinase. (**A**) Localization of Rho-kinase in COS7 cells. COS7 cells were transfected with myc–Rho-kinase and HA–RhoA or HA–RhoA^{V14} as indicated. The particulate fraction was immunoblotted for the presence of myc–Rho-kinase. (**B**) Immunofluorescence analysis of NIH 3T3 cells. NIH 3T3 cells were transfected with myc–Rho-kinase and double-labeled with anti-myc antibody (**a**) and TRITC–phalloidin (**b**). An arrowhead indicates the transfected cells. The results shown are representative of three independent experiments.

Rho-kinase and induces a conformational change, leading to activation of the kinase toward selective substrates such as the myosin binding subunit. To further analyze the mode of activation of Rho-kinase by RhoA, we transfected the cDNA of Rho-kinase into COS7 cells and immunoprecipitated it. We could demonstrate that the recombinant Rho-kinase showed autophosphorylation and kinase activity toward exogenous substrates (data not shown). However, activated RhoA stimulated kinase activity <2-fold. Although the exact reason for this poor stimulation is not clear, it may be due to partial activation of Rho-kinase in COS7 cells during transfection, since COS7 cells are transformed and Rho-mediated signaling pathways are in an active state. Further studies are necessary to understand the precise mechanisms that account for the mode of activation of Rho-kinase by Rho.

Relation to myotonic dystrophy kinase

The catalytic domain of Rho-kinase is 72% homologous to that of myotonic dystrophy kinase, which is the product of the gene that causes myotonic dystrophy (Brook *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*, 1992). Myotonic dystrophy is an autosomal dominant multisystem disease that is characterized by muscle weakness, atrophy and myotonia (Harper, 1989). Myotonic dystrophy kinase has a catalytic domain in the N-terminal portion, a coiled coil domain in the middle portion and a transmembrane domain in the C-terminal portion. Thus myotonic dystrophy kinase has a molecular design similar to Rho-kinase, except for the transmembrane domain. It would be interesting to examine whether activated RhoA interacts with myotonic dystrophy kinase, which is underway in our laboratory. Although the catalytic properties of myotonic dystrophy kinase have not been compared with Rho-kinase, they may share similar catalytic properties, since their catalytic domains are very similar.

Catalytic properties of Rho-kinase

The functions of kinases are defined by their activators and physiological substrates. The catalytic domain of PKN is highly related to that of PKC (Nishizuka, 1988; Mukai and Ono, 1994). The substrate specificity of PKN appears to be similar to that of PKC (Mukai *et al.*, 1994). Among S6 peptide, αPKC peptide and myelin basic protein, αPKC peptide is the best substrate for PKN (data not shown). In contrast, among them S6 peptide is the best substrate for Rho-kinase, as shown in Figure 4A. Thus the substrate specificity of Rho-kinase differs from that of PKN, suggesting that they have distinct functions. We have also shown that the myosin binding subunit is the preferred substrate for Rho-kinase among cytoskeletal regulatory proteins so far examined (Figure 5). Although GTPγS·GST–RhoA stimulates the kinase activity of Rho-kinase toward S6 peptide at most 4-fold, it stimulates kinase

activity toward the myosin binding subunit >15-fold, suggesting that GTP·Rho dramatically stimulates kinase activity toward the preferred substrates. We are now investigating whether the myosin binding subunit serves as a physiological substrate for Rho-kinase.

Thus, upon stimulation GTP·RhoA seems to interact with unique subsets of protein kinases, such as Rho-kinase and PKN. Such a diversity of Rho-interacting proteins may account for the diversification of downstream pathways from Rho to induce appropriate cytoskeletal responses to extracellular signals, such as lysophosphatidic acid and specific growth factors. It is essential to identify physiological substrates for Rho-kinase and PKN and to examine how these kinases are activated during the action of extracellular signals such as lysophosphatidic acid to gain a better understanding of the mode of action of Rho.

Materials and methods

Materials and chemicals

GST–RhoA, GST–RhoA^{A37}, GST–Rac1 and GST–H-Ras were purified and loaded with guanine nucleotides as described previously (Shimizu *et al.*, 1994b). Chicken cytoskeletal proteins (vinculin, talin, metavinculin, caldesmon, filamin and α-actinin) were kindly provided by Dr T.Endo (Chiba University, Japan), vimentin by Dr M.Inagaki (The Tokyo Metropolitan Institute of Gerontology, Japan) and MAP-4 by Dr H.Murofushi (University of Tokyo, Japan). Rat myosin binding subunit was expressed as a fusion protein with maltose binding protein in *Escherichia coli* and purified (Shimizu *et al.*, 1994a). pGEM-HA was kindly provided by Dr S.Orita (Shionogi Institute for Medical Science, Japan). [^{35}S]GTPγS was purchased from DuPont New England Nuclear. [γ-^{32}P]ATP and [^{35}S]methionine were purchased from Amersham Corp. All materials used in the nucleic acid study were purchased from Takara Shuzo Co. Ltd (Kyoto, Japan). Other materials and chemicals were obtained from commercial sources.

Purification of p164

A homogenate of bovine brain gray matter (190 g) was prepared and centrifuged at 20 000 *g* for 30 min at 4°C as described (Yamamoto *et al.*, 1995). The precipitate was suspended in 360 ml homogenizing buffer [25 mM Tris–HCl, pH 7.5, 5 mM EGTA, 1 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride, 1 mg/l leupeptin, 10% sucrose] to prepare the crude membrane fraction. The proteins in this fraction were extracted by addition of an equal volume of homogenizing buffer containing 4 M NaCl. After shaking for 1 h at 4°C the membrane fraction was centrifuged at 20 000 *g* for 1 h at 4°C. The supernatant was dialyzed against buffer A (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$) three times. Solid ammonium sulfate was then added to a final concentration of 40% saturation. The 0–40% precipitate was dissolved in 16 ml buffer A, dialyzed against buffer A three times and used as the membrane extract. The membrane extract was passed through a 1 ml glutathione–Sepharose column. The flow-through fraction was loaded onto a 1 ml glutathione–Sepharose column containing 24 nmol GTPγS·GST–RhoA. Proteins were eluted by addition of 10 ml buffer A containing 1% CHAPS and fractions of 1 ml each were collected. p164 protein appeared in fractions 2–10. The sample (fractions 3–10) was diluted with an equal volume of buffer A and subjected to a Mono Q 5/5 column equilibrated with buffer A. After washing with 10 ml buffer A proteins were eluted with a linear gradient of NaCl (0–0.5 M) in 15 ml buffer A and fractions of 0.5 ml each were collected. p164 protein appeared as a single peak in fractions 10–12.

Overlay assay

The method is a modified form of that described previously (Manser *et al.*, 1992). Briefly, the sample was subjected to 6% SDS–PAGE and blotted onto a nitrocellulose membrane. The membrane was incubated at 4°C for 5 min with buffer B (25 mM HEPES–NaOH, pH 7.0, 0.5 mM $MgCl_2$, 0.05% Triton X-100) containing 6 M guanidium hydrochloride, followed by incubation for 3 min in buffer B containing 3 M guanidium hydrochloride. This was repeated four times. The membrane was agitated for 10 min and an equal volume of buffer B was added five times sequentially for 10 min each. The membrane was soaked in buffer B, transferred to phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 0.1% Triton X-100, 0.5 mM $MgCl_2$ and 5 mM DTT, then immersed for 10 min in 0.5 ml GAP buffer (25 mM HEPES–NaOH, pH 7.0, 2.5 mM DTT, 5 mM $MgCl_2$, 0.05% Triton X-100, 100 mM GTP) containing [^{35}S]GTPγS·GST–RhoA or [^{35}S]GTPγS·GST–RhoA^{A37}. The membrane was washed three times with PBS containing 25 mM HEPES–NaOH, pH 7.0, 5 mM $MgCl_2$, 0.05% Triton X-100, dried and exposed to X-ray film.

Protein kinase assay

The kinase reaction was carried out in 50 μl kinase buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 0.06% CHAPS) containing 2 μM [γ-^{32}P]ATP (600–800 GBq/mmol) and purified p164 (10 ng protein) with or without exogenous substrates. After an incubation for 10 min at 30°C the reaction mixtures were boiled in SDS sample buffer and resolved by SDS–PAGE to examine autophosphorylation of p164 and phosphorylation of cytoskeletal regulatory proteins. The radiolabeled bands were visualized by autoradiography. To examine phosphorylation of S6 peptide, PKCα peptide and myelin basic protein the reaction mixtures were spotted onto Whatman p81 paper. Incorporation of ^{32}P into the substrates was assessed by scintillation counting.

Peptide sequencing

Purified p164 was resolved by SDS–PAGE and transferred to a polyvinylidene difluoride membrane. The band corresponding to p164 was digested by lysyl endopeptidase, *Achromobacter* protease I and endoproteinase Asp-N (Iwamatsu, 1992). The resulting peptides were fractionated by C18 column chromatography and subjected to amino acid sequencing for identification. Thirty-seven internal sequences from the peptides were obtained.

Cloning of p164 cDNA

To obtain cDNA clones encoding p164, a bovine brain cDNA library (1.2×10^6 independent plaques in total) was screened with degenerate oligonucleotide probes based on the partial amino acid sequences determined from purified p164 (indicated by double underlining in Figure 6A). Hybridization to screen the library was as described (Sambrook *et al.*, 1989). The cDNA inserted into λgt10 phage DNA was cloned into pBluescript II SK(–) (Alting-Mees and Short, 1989) for nucleotide sequencing with an ABI DNA sequencer 373S.

Antibody production and immunoblot analysis

Rabbit polyclonal antibodies against peptides corresponding to the partial amino acid sequences of bovine Rho-kinase (CAFLTDREVRLGRNG and CKRQLQERFTDLEK) were generated and purified. Immunoblot analysis of Rho-kinase was carried out as described (Harlow and Lane, 1988).

Interaction of activated RhoA with recombinant Rho-kinase in a cell-free system

To obtain the *in vitro* translated coiled coil domain of Rho-kinase pGEM-HA-Rho-kinase was constructed as follows. The 2.2 kb cDNA fragment encoding Rho-kinase (amino acids 421–1137) was amplified by PCR from the Rho-kinase cDNA clone with primers 5′-ATAAGGATCCCT-ACTAAGTGACTCTCCATCTTG-3′ and 5′-TATAGGATCCTTAACT-GCCTATACTGGAACTATCC-3′. The cDNA fragment was cloned into the *Bam*HI site of pGEM-HA. The coiled coil domain was translated *in vitro* using the TNT T7 coupled reticulocyte lysate system (Promega) under the conditions described in the instruction manual. GST small G proteins loaded with guanine nucleotides (0.75 nmol each) were immobilized onto 31 μl glutathione–Sepharose 4B beads and washed with 310 μl (10 vol) buffer A. The immobilized beads were added to 30 μl of *in vitro* translated mixture and gently mixed for 1 h at 4°C in the presence of 1 mg/ml BSA. The beads were washed six times with 102 μl (3.3 vol) buffer A and the bound proteins were eluted with GST small G proteins by addition of 102 μl (3.3 vol) buffer A containing 10 mM glutathione three times. The first eluates were subjected to SDS–PAGE and vacuum dried, followed by autoradiography.

Localization of Rho-kinase and RhoA in COS7 cells

To express myc epitope-tagged Rho-kinase, the cDNA encoding Rho-kinase was cloned into pEF-BOS-myc to yield pEF-BOS-myc-Rho-kinase (Mizushima and Nagata, 1990). The plasmids pEF-BOS-myc-Rho-kinase and pEF-BOS-HA-RhoA or pEF-BOS-HA-RhoA^{V14} were transfected into COS7 cells to express myc–Rho-kinase and HA–RhoA or HA–RhoA^{V14} (Mukai *et al.*, 1994). After 40 h the cells were harvested,

suspended in lysis buffer (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 2.5 μg/ml leupeptin, 0.15 M NaCl) and homogenized in a Dounce homogenizer. Nuclei and debris were pelleted by centrifugation at 4000 *g* for 5 min. The post-nuclear supernatant was centrifuged at 100 000 *g* for 30 min. The cytosol and particulate fractions were subjected to immunoblot analysis using antibodies to the HA (12CA5) and myc epitopes (9E10).

Immunofluorescence analysis of NIH 3T3 cells expressing myc-tagged Rho-kinase

NIH 3T3 cells were transfected with pEF-BOS-myc-Rho-kinase (Ridley *et al.*, 1992). For localization of myc–Rho-kinase and actin filaments cells were fixed in 3.7% paraformaldehyde in PBS for 20 min. The fixed cells were incubated for 10 min with 50 mM ammonium chloride in PBS and permeabilized with PBS containing 0.2% Triton X-100 for 10 min. After being soaked in 10% FCS/PBS for 30 min the cells were treated with an anti-myc antibody (9E10) in 10% FCS/PBS for 1 h. The cells were then washed with PBS three times, followed by incubation with an anti-mouse IgG and TRITC–phalloidin in 10% FCS/PBS for 1 h. After being washed with PBS three times the cells were examined using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Other procedures

SDS–PAGE was performed as described (Laemmli, 1970). Protein concentrations were determined with BSA as the reference protein as described (Bradford, 1976). The BLAST program was used for a protein homology search (Altschul *et al.*, 1990). The COILS program was used for prediction of the coiled coil region (Lupas *et al.*, 1991).

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After we submitted this paper, the truncated form of a similar kinase was reported [Leung *et al.* (1995) *J. Biol. Chem.*, **270**, 29051–29054].