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Functions of a Rho-specific guanine nucleotide-exchange factor in neurite retraction: Possible role of a proline-rich motif of KIAA0380 in localization."

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Running title: Functions of KIAA0380 in neurite retraction
Summary

The Rho/Rho-kinase signaling pathway plays an essential role in neurite retraction and cell rounding in response to $G_{12/13}$-coupled receptor activation in neuronal cells. The Rho guanine nucleotide exchange factor (RhoGEF) involved in these processes has not been identified. To monitor the activation state of Rho-kinase, we developed a vimentin head/Rho-kinase chimera which is intramolecularly phosphorylated in a Rho-dependent manner at Ser71 of the fused vimentin head. Using this system, we identified a clone termed KIAA0380, which contains the $G_{\alpha_{12/13}}$-binding domain as well as a tandem of the DH/PH domain, as an activator of Rho/Rho-kinase signaling. Molecular dissection analyses revealed that a proline-rich motif C-terminally adjacent to DH/PH domain is essential for plasma membrane localization of KIAA0380 and cortical actin reorganization followed by cell rounding. In contrast, the DH/PH domain of KIAA0380 is localized in the cytoplasm where it activates Rho/Rho-kinase and induces stress fiber formation, consistent with results using p115 RhoGEF which has a similar structure to KIAA0380 but lacks a proline-rich motif. These results suggest that upon stimulation, KIAA0380 translocates to the plasma membrane via the proline-rich motif and there activates Rho/Rho-kinase signaling. In neuroblastoma Neuro2a cells, KIAA0380 was observed in the tips of neurites, a location where cortical actin reorganization is induced upon stimulation with lysophosphatidic acid (LPA). Ectopic expression of the N-terminal fragment inhibited LPA-induced neurite retraction of Neuro2a cells. These results suggest that KIAA0380 plays an important role in neurite retraction through Rho-dependent signaling.
Introduction

It is well established that Rho, Rac and Cdc42, three members of the Rho family of small GTPases, control both the organization of the actin cytoskeleton and signal transduction pathways leading to gene transcription. In fibroblasts, Rho controls the assembly of actin stress fibers and associated focal adhesion complexes, while Rac and Cdc42 control the formation of lamellipodia and filopodia respectively (1-4). In addition to these effects, the three GTPases have been reported to trigger a number of additional cellular activities (1-4). For example, Rho is required for G1 cell cycle progression, activates the SRF transcription factor and plays a role in the cell cycle during cytokinesis.

Cytoskeletal changes mediated by Rho vary between cell types. In neuronal cell lines, Rho induces the formation of a cortical shell of F-action that mediates cytoskeletal contraction (5), which is thought to underlie growth cone collapse, neurite retraction and rounding cell body in response to lysophosphatidic acid (LPA) (5-10). However, the biochemical relationship between these varied responses remained to be clarified.

Like other small GTPases, Rho is thought to act as a molecular switch to control intracellular signal transduction pathways; it exists in either an inactive (GDP-bound) or an active (GTP-bound) conformation, and regulatory proteins that control this GDP/GTP cycle include over 15 distinct guanine nucleotide exchange factors (GEFs), around 10 GTPase-activating proteins (GAPs) and at least two guanine nucleotide dissociation inhibitors (GDIs) (11-13). Many GEFs for Rho family proteins were discovered by virtue of their ability to transform NIH3T3 cells when overexpressed or when activated by truncation. All these proteins share a 250-amino acids stretch of significant sequence similarity with Dbl, termed Dbl-homology (DH) domain, adjacent to a pleckstrin-homology (PH) domain (11). The DH domain was shown to be responsible for nucleotide exchange activity toward Rho family GTPases (14-16).

Several Rho-targets have been identified, including Rho-kinase. This kinase regulates the phosphorylation of myosin light chain (MLC) of myosin II by direct phosphorylation of MLC and by inactivation of myosin phosphatase through phosphorylation of MBS (17-19). In addition to MLC and MBS, Rho-kinase phosphorylates the ERM family proteins (ezrin, radixin, moesin) and adducin both in vitro and in vivo (20-22). Rho-kinase has been shown to regulate the formation of actin stress fibers and focal adhesions (23-25), smooth muscle contraction (26), myosin fiber organization and c-fos
expression (19), efficient separation of glial filaments (27), cytokinesis (27, 28) and neurite retraction (29, 30). In spite of the physiological importance of Rho-kinase, direct detection of the in vivo activation state of Rho-kinase has not been feasible.

We designed a novel in vivo detection system for the state of Rho-kinase activation and used this system to identify the novel activator for Rho/Rho-kinase signaling, KIAA0380, which was originally isolated from a human brain cDNA library in a random cloning project (31). After confirming that KIAA0380 actually functions as a Rho-specific GEF in vitro and in vivo, cell biological characterization of KIAA0380 was done. KIAA0380 induced actin reorganization followed by cell rounding in various types of cells, and induced neurite retraction and cell rounding in mouse neuroblastoma Neuro2a cells. Since KIAA0380 is composed of various functional domains that are commonly found in signaling molecules, we prepared several mutants and analyzed the physiological significance of the functional domains. Interestingly, in addition to DH/PH domain, a proline-rich motif which is C-terminally adjacent to the DH/PH domain is also essential for plasma membrane location of KIAA0380, cortical actin reorganization and cell rounding. Our data suggest that the proline-rich motif of KIAA0380 is involved in localization to the cell membrane and to the biological activity of KIAA0380. In Neuro2a cells, the N-terminus fragment of KIAA0380 inhibited LPA-mediated neurite retraction. Immunological analysis revealed that KIAA0380 is well expressed in Neuro2a cells, but another Go12/13-binding p115 RhoGEF, which is also termed Lsc (32), was not detected. Taken together, these findings suggest that in neuronal cells, KIAA0380 functions as a RhoGEF at the cell periphery and regulates Go12/13-coupled receptor-mediated Rho activation, an event essential for neurite retraction and growth cone collapse.
Experimental Procedures

Materials

The following constructs were kind gifts from colleagues; Dr. K. Kaibuchi (Nara Inst. Sci. Technol., Japan), cDNAs of Rho-kinase and the mutants; Drs. T. Nagase (Kazusa DNA Inst., Japan) and T. Kozasa (University of Texas), KIAA0380 cDNA; Dr. A. Hall (University College London, UK), pRK5-Myc plasmids harboring L63RhoA, N19Rho, L61Rac and L61Cdc42; Dr. T. Kiyono (our institute), anti-Myc antibody (9E10); Dr. G. Bollag (Onyx Pharmaceuticals, CA), pEXV-Myc-p115 RhoGEF and anti-p115 RhoGEF antibody. Mouse Neuro2a neuroblastoma cells were kindly provided by Dr. Y. Takeda (Tokyo Metropolitan Inst. Gerontol.). Anti-FLAG-tag antibody (M2) was purchased from Kodak Inc.. Other materials and chemicals were obtained from commercial sources.

Plasmid construction

pRK5-Myc-VH-Rho-kinase and pRK5-Myc-VH-Rho-kinase(KDTT), which contains mutations at the ATP-binding and Rho-binding sites of the kinase and thereby functions as a kinase-negative version (29), were obtained by fusing with the intermediate filament protein vimentin head domain (aa2-87) with respective Rho-kinase cDNAs and constructed into pRK5 vector containing Myc tag. The cDNA fragments of KIAA0380-FL (aa1-1522), -DH (aa735-958), -DH/PH-C (aa1485-1522), -DH/PH-Pro (aa735-1119), -RGS-DH/PH(aa301-1080), -DH/PH (aa735-1080), -DH (aa735-958), -N (aa1-592) and -C (aa1056-1522) were subcloned into pRK5-Myc vector. For site-directed mutagenesis, QuickChange site-directed mutagenesis kits (Stratagene) were used. All constructs were verified by DNA sequencing.

Expression and purification of recombinant proteins

GTPases, KIAA0380-RGS-DH/PH and KIAA0380-C were expressed in E. coli as glutathione S-transferase (GST) fusion proteins and purified on glutathione-sepharose beads. The recombinant proteins were released from the beads by cleavage with human thrombin. Protein concentration was determined by the method of Bradford (33) and purity of the protein preparations was confirmed on Coomassie Blue-stained SDS-polyacrylamide gels.
Preparation and characterization of an anti-KIAA0380 Antibody

KIAA0380-C fragment expressed in *E. coli* was used as an antigen. A rabbit polyclonal antibody specific for KIAA0380-C fragment was produced and characterization was carried out as described elsewhere (34). Western blot analysis was done and the immunoreactive bands were visualized by making use of a horseradish-peroxidase-conjugated anti-rabbit antibody (Amersham) and the ECL western blotting detection system (Amersham).

Cell culture, microinjection, transfection and immunofluorescence

Swiss 3T3, COS7 and Neuro2a cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FCS). MDCKII cells were maintained in DMEM containing 10% calf serum. COS7 and Neuro2a cells were transfected by the Lipofectamine method (Gibco-BRL). After 24 h, transfected COS7 cells were serum-starved for 16 h and then harvested. Transfected Neuro2a cells on poly-D-lysine-coated coverslips were maintained in a serum-starved condition for 24 h and then harvested. Swiss 3T3 and MDCKII cells nuclei were microinjected with pRK5-Myc vectors containing wild type or mutants of KIAA0380 and p115RhoGEF, as described (35). Cells were fixed in 3.7% formaldehyde in PBS for 15 min then were subsequently permeabilized with 0.2% Triton-X 100 for 5 min. Exceptionally, fixation was done using 10% trichloroacetic acid to detect RhoA. After primary antibody incubation at 37°C for 2 h, the cells were incubated with a combination of the secondary antibody and rhodamine-conjugated phalloidin (Molecular Probes) to detect F-actin. To detect the transfected proteins, 9E10 (a mouse monoclonal anti-myc antibody) and FITC-labeled anti-mouse IgG were used. For detection of KIAA0380, affinity-purified anti-KIAA0380 antibody was used as the primary antibody, and Alexa 488 anti-rabbit IgG (Molecular Probes) as the secondary one. Anti-RhoA (Santa Cruz), anti-tubulin (Sigma) and anti-vimentin mouse monoclonal antibodies were used as primary antibodies to detect RhoA, tubulin and vimentin, respectively. Cells were analyzed using a confocal microscope (Olympus, LSM-GB200).

In vitro and in vivo analyses of GDP/GTP exchange activity of KIAA0380

In vitro GDP/GTP exchange activity of recombinant Rho, Rac or Cdc42 (10 pmol) was measured, as described (36) in the presence or absence of recombinant GST-KIAA0380-RGS-DH/PH
fragment (20 pmol). Analysis of guanine nucleotides bound to Rho, Rac and Cdc42 was made, as described (37). Briefly, COS7 cells expressing FLAG-tagged Rho, Rac or Cdc42, with or without Myc-KIAA0380, were labeled with \[^{32}P\]Pi at 0.1 mCi/ml for 4 h then the cells were lysed in lysis buffer containing 20 mM Tris/HCl (pH7.5), 150 mM NaCl, 20 mM MgCl\(_2\), 1 mM Na\(_3\)VO\(_4\), 0.5 % Triton X-100, 1 mM PMSF and 10 \(\mu\)g/ml aprotinin. FLAG-tagged GTPases were then immunoprecipitated with M2 anti-FLAG antibody. After denaturation of the proteins, eluted nucleotides were analyzed by polyethyleneimine thin-layer chromatography. Guanine nucleotides were detected and quantitated using the BAS 2500 system (Fuji Film, Tokyo, Japan).

**Immunoprecipitation**

Neuro2a and COS7 cells transiently expressing KIAA0380 or p115 RhoGEF were harvested with of RIPA buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1.0 % NP-40, 0.5 % deoxycholate and 0.1 % SDS. Insoluble material was removed by centrifugation at 4\(^\circ\)C for 10 min at 10,000 x g, and 50 \(\mu\)l of lysate (50-90 \(\mu\)g of protein) was used for each assay. KIAA0380 and p115 RhoGEF were immunoprecipitated, using specific antibodies. After washing the precipitates three times with RIPA buffer, the precipitates were subjected to SDS-PAGE (7.5 % gel) and proteins were transferred to nitrocellulose filters. Western blotting was done using anti-KIAA0380 and anti-p115 RhoGEF antibodies.
Results

Development of a system to detect the activation state of Rho-kinase.

The head domain of vimentin is phosphorylated specifically at Ser71 by Rho-kinase, and the phosphorylation state of vimentin can be detected using a site- and phosphorylation state-specific antibody, TM71 (38-40). Such being the case, Ser71 is useful for monitoring the vimentin head phosphorylation state in vivo by Rho-kinase. Based on this characteristic feature, we attempted to establish a chimera construct in which the vimentin head domain (aa2-87) is fused to the N-terminus of Rho-kinase (Fig. 1A). Since Rho activates the Rho-kinase pathway in vitro and in vivo, we first asked whether Rho could activate VH-Rho-kinase and phosphorylate the attached vimentin head domain at Ser71. pRK5-Myc-VH-Rho-kinase was co-transfected into COS7 cells using various Rho, Rac and Cdc42 constructs. Phosphorylation of VH-Rho-kinase was monitored by western blotting, using TM71. As shown in Fig. 1B, only the active version of Rho (L63Rho) strongly induced phosphorylation of vimentin-Ser71, whereas an inactive version of Rho (N19Rho) and constitutively active Rac and Cdc42 did not do so. These results indicate that the phosphorylation at Ser71 of vimentin head of VH-Rho-kinase is an active Rho-dependent event.

As illustrated in Fig. 1A, there are at least two possible molecular mechanisms for Ser71 phosphorylation, one is in intramolecular and the other is intermolecular. To determine which molecular mechanism underlies the observed Ser71-phosphorylation, we analyzed the inactive version of VH-Rho-kinase, KDTT. As shown in Fig. 1C, fetal bovine serum induced Ser71 phosphorylation of wild type VH-Rho-kinase but not KDTT. Even when constitutively activated Rho-kinase (CAT) was co-transfected with KDTT, phosphorylation of Ser71 did not occur (Fig. 1C), which strongly suggests that endogenous Rho-kinase cannot interact with the vimentin head of VH-Rho-kinase. In these experiments, phosphorylation of endogenous vimentin at Ser71 was detected, as shown in Fig. 1C. Taken together, we considered that the phosphorylation of Ser71 of VH-Rho-kinase is an intramolecular event.

Since ectopically expressed constitutively active Rho-kinase(CAT) localizes diffusely in the cytoplasm and is no longer regulated by RhoGEF/Rho, it can phosphorylate endogenous vimentin (Fig. 1C). On the other hand, serum stimulation did not induce endogenous vimentin phosphorylation (Fig. 1C), perhaps because the intracellular localization of activated Rho-kinase is restricted by
RhoGEF/Rho, thus the kinase may not interact with and phosphorylate endogenous vimentin.

Identification of KIAA0380 as an activator for Rho-Rho-kinase signaling.

As our newly designed Rho-kinase activity detection system proved useful to monitor the activation state of the kinase, this system may be able to detect novel Rho-kinase activators. KIAA0380 was first isolated from a human brain cDNA library in a random cloning project (31). Since this activator contains a tandem of the DH/PH domain and functions as a RhoGEF in vitro (41), we asked if KIAA0380 functions as an in vivo activator for Rho-kinase. As shown in Fig. 2A, KIAA0380 strongly induced phosphorylation of Ser71 of VH-Rho-kinase, to an extent similar to that seen with p115 RhoGEF. In contrast, the deletion mutant lacking the DH domain (KIAA0380•DH) did not phosphorylate Ser71. KIAA0380 most likely activates Rho-kinase through the direct activation of Rho.

Since KIAA0380 activates VH-Rho-kinase in vivo and consequently induces autophosphorylation of the kinase at Ser71, KIAA0380 may directly activate Rho. To determine if the KIAA0380-induced Ser71-phosphorylation occurs through activation of Rho signaling by KIAA0380 and not through Rho-independent events, we wanted to determine if KIAA0380 would directly activate Rho both in vitro and in vivo. As for in vitro experiments, recombinant Rho, Rac and Cdc42 were first complexed with nonradioactive GDP and then incubated with [35S]GTPγS in the presence or absence of GST-fused KIAA0380-RGS-DH/PH. As shown in Fig. 2B, KIAA0380-RGS-DH/PH accelerated the binding of GTPγS to Rho but not to Rac or to Cdc42, findings consistent with reported data (41).

Since some Dbl family proteins show different substrate specificities in vitro and in vivo (42, 43), we wanted to determine if KIAA0380 would activate Rho in vivo. The pRK5-Flag vector harboring Rho, Rac or Cdc42 was introduced into COS7 cells. When Rho, Rac and Cdc42 were expressed by themselves, the bound GTP accounted for 16.4%, 5.8% and 5.9%, respectively. We then examined the effects of KIAA0380 regarding activation of these GTPase. As shown in Fig. 2C, coexpression of KIAA0380 but not Rac and Cdc42, led to a increase in the GTP-bound form of Rho, up to 34.4%. These results of in vivo experiments indicate that KIAA0380 is a physiological Rho-specific GEF and also confirm reliability of our system for detecting Rho-kinase activity.
Overexpression of KIAA0380 induces actin reorganization and marked rounding of Swiss 3T3

RhoGEFs are important regulators of Rho-dependent actin polymerization. To confirm the involvement of KIAA0380 in actin reorganization, we microinjected pRK5-Myc-KIAA0380 into Swiss 3T3 cells and 3 h later, KIAA0380-expressing cells were identified by staining the Myc-epitope. F-actin organization was examined after staining with phalloidin. Cells expressing KIAA0380 showed a marked cell rounding and actin staining was strong underneath plasma membranes of the round cell bodies (Fig. 3A-C). On the other hand, p115 RhoGEF, a Rho-specific GEF structurally and highly homologous to KIAA0380, did not induce cortical actin reorganization followed by cell rounding but did induce stress fibers (Fig. 3D-F).

Determination of functionally important domains of KIAA0380

In addition to a tandem of DH and PH domains, KIAA0380 exhibits characteristic structural features such as the PDZ domain involved in protein-protein interaction and the RGS motif interacting with \( \alpha \) subunits of G\(_{12} \) and G\(_{13} \) (31, 44). To determine the physiological significance of each structural domain, we prepared a variety of truncation and deletion mutants of KIAA0380, as shown in Fig. 4A, and transiently expressed them in Swiss 3T3 cells. As depicted in Fig. 4B, the results show that the DH domain and also the PH domain are essential for actin reorganization since the DH domain alone does not induce reorganization. Moreover, it is notable that the DH/PH domains per se do not induce full contraction of cells; a proline-rich motif (aa1081-1119) adjacent to the PH domain is required for KIAA0380-induced cell rounding.

KIAA0380 induces cytokinetic contraction in MDCKII cells, and inhibits neurite extension of Neuro2a cells.

The contraction of polymerized F-actin appeared to be strong and the fully rounded phenotype was observed even in MDCKII cells which have tight cell-cell attachments (Fig. 5A-F). Since KIAA0380 is predominantly expressed in the brain, and cortical actin reorganization followed by cell
rounding is a characteristic feature of neuronal cells in which Rho-Rho-kinase signaling is activated, we next examined the effects of KIAA0380 on the morphology of Neuro2a cells. We transiently overexpressed KIAA0380 or L63Rho in Neuro2a cells and examined morphology of the cells expressing the respective proteins. After transfection, the cells were incubated in serum-free DMEM for 16 h. Transfected cells were identified by staining the Myc epitope and F-actin organization was examined. In serum-free medium, nontransfected cells had flattened cell bodies and extended neurites (Fig. 5G-I). While cells expressing KIAA0380 showed marked cell rounding and extended neurites were never apparent. In this transfectant, strong actin staining was evident in the rounded cell bodies. These results clearly indicate that activation of Rho signaling by KIAA0380 is sufficient to form cortical shells of F-actin that mediate cytoskeletal contraction and cell rounding.

Expression of KIAA0380 in Neuro2a cells

As KIAA0380 is highly expressed in the brain, it may play a role in neuronal cellular processes regulated by Rho. To elucidate related physiological functions, we developed a rabbit polyclonal antibody (α-KIAA0380-C) against the bacterially synthesized C-terminal fragment of KIAA0380 (aa1056-1522). We first analyzed KIAA0380 protein expression in Neuro2a cells by immunoprecipitation in combination with western blotting. As shown in Fig. 6 (lane 2), a relatively high level expression of KIAA0380 was observed in Neuro2a cells. In contrast, p115 RhoGEF, a KIAA0380-related RhoGEF containing a RGS motif and can associate with Gα12/13, was not detected in Neuro2a (lane 4). These observations are consistent with findings that KIAA0380 mRNA is highly expressed in the brain but to a lesser extent in many other tissues (31, 44). Since Neuro2a cells can be rapidly induced to produce neurites after serum withdrawal and do not express p115 RhoGEF, this cell line is pertinent to characterize KIAA0380 functions in neurite retraction.

Immunocytochemical analyses of KIAA0380 in Neuro2a cells

In the next set of experiments, immunocytochemical analysis was made to determine the intracellular localization of endogenous KIAA0380. Fig. 7A-C shows that endogenous KIAA0380 is localized in the nucleus and cell body, in addition to neurites of Neuro2a cells, and without any
characteristic pattern. However, once the cells are stimulated with 1µM LPA for 10 min, the staining pattern of KIAA0380 changes. In some cells, KIAA0380 was intensely stained in some tips of neurites where actin is reorganized (Fig. 7D-F; arrowheads), and in other cells KIAA0380 is enriched in retracted neurites (Fig. 7G-I; arrowheads). KIAA0380 was also enriched in cell peripheral areas where the F-actin shell is formed (Fig. 7G-I; arrows). Since KIAA0380 is considered to be a Rho-specific GEF, we compared the intracellular KIAA0380 localization to that of endogenous Rho. As shown in Fig. 7J-L, Rho was enriched in neurite tips where KIAA0380 had accumulated, thereby suggesting direct interactions of KIAA0380 with Rho in growth cones. To determine if KIAA0380 interacts with other cytoskeletal proteins, we co-stained KIAA0380 with tubulin and vimentin. As shown in Fig. 7N and Q, tubulin and vimentin are distributed throughout the cytoplasm but not concentrated in neurite tips. KIAA0380 appears to co-localize with microtubules and intermediate filaments at neurite tips (Fig. 7M-R). The physiological relation between KIAA0380 and these cytoskeletons remains to be clarified. KIAA0380 was also observed in the nuclei in interphase cells as is the case with Ect2 (45), although the dynamic change of distribution was not observed during cytokinesis (data not shown).

**Inhibition of LPA-induced neurite retraction of Neuro2a by KIAA0380 fragments**

KIAA0380 mutants without a complete DH/PH domain cannot catalyze guanine nucleotide exchange of Rho (Fig. 1B and Fig. 2A). In neuronal cells, LPA activates Rho to induce growth cone collapse and neurite retraction through a G_{12/13}-initiated pathway that involves protein-tyrosine kinase activity (9, 46, 47). p115 RhoGEF has been shown to activate Rho in a G_{13}-dependent manner *in vitro* (48). Since p115 RhoGEF is not detectable in Neuro2a cells, KIAA0380 may have an important role in G_{12/13}/Rho-dependent signals which result in neurite retraction. To investigate the role of KIAA0380 in the signaling pathway utilized by LPA, we used a KIAA0380-N fragment. Of interest, expression of KIAA0380-N (1.0 µg) in Neuro2a cells significantly inhibited cell rounding by LPA stimulation (Fig. 8). A dose-dependent inhibition by KIAA0380-N of neurite retraction was evident between 0 and 1.0 µg of the plasmid used. However, the inhibition of LPA-induced neurite retraction by KIAA0380-N was not complete, since more than 1.0 µg of the KIAA0380-N cDNA led to cytotoxicity (data not shown).
Discussion

The Rho family of proteins has important roles in actin cytoskeletal reorganization and cell-matrix interactions and all are essential determinants of neurite extension and retraction. Activation of these small GTPases in response to extracellular stimuli is regulated by their regulatory proteins such as GEF, GDI and GAP.

Based on previous studies, it is most likely that generation of the actin-based contractile forces is required for neurite retraction (6, 7). A great deal of progress has been made in elucidating biochemical pathways governing neurite extension and retraction of neuronal cells. The activation of a certain G protein-coupled receptor, such as the LPA, thrombin and prostaglandin EP3 receptors, was shown to induce Rho-dependent neurite retraction in several differentiated neuronal cell lines (7, 49-52). Thereafter, the G₁₂ family of heterotrimeric G proteins, defined by Gα₁₂ and Gα₁₃, were found to be involved in Rho-dependent actin stress fiber formation and focal adhesion assembly, as determined by microinjection analyses (53). Using this approach, Gα₁₂ and Gα₁₃ were seen to activate Rho, but not Gα₂ in Swiss 3T3 cells. The constitutively activated versions of both Gα₁₂ and Gα₁₃ were found to induce a range of Rho-dependent responses, not only stress fiber formation but also SRF (54) and phospholipase D activation (55, 56) in fibroblasts, and neurite retraction in neuronal cell lines (57). Further analyses revealed that LPA-induced Rho activation is mediated by Gα₁₃ in fibroblasts, while thrombin appears to act through Gα₁₂ (47, 58).

Although the physiological RhoGEF which links between Gα₁₂/Gα₁₃ and Rho is unknown, p115 RhoGEF (also termed Lsc) was found to bind to Gα₁₂ and Gα₁₃ through their RGS domain and to be selectively activated by Gα₁₃ in vitro (48). To date, two kinds of RhoGEF containing Gα₁₂/Gα₁₃-interactive RGS domains have been identified; one is p115 RhoGEF and the other is KIAA0380. Since growth cone collapse, neurite retraction and cell rounding in neuronal cells are Gα₁₂- and Gα₁₃-dependent processes, we assume that Rho-specific GEF containing the RGS domain which can interact with Gα₁₂ and/or Gα₁₃ is a possible candidate as regulator of neurite retraction. KIAA0380 is structurally homologous to p115 RhoGEF and has the Gα₁₂ and Gα₁₃-interactive RGS motif. Northern analysis revealed that p115 RhoGEF is highly expressed in peripheral blood leukocytes, thymus and spleen, but hardly detectable in the brain (32); we never detected p115 RhoGEF protein in Neuro2a cells. In contrast, KIAA0380 is dominantly expressed in the brain, at the mRNA level and the protein
is abundant in Neuro2a cells. It may be that KIAA0380 can be activated directly by Gα_{12} and/or Gα_{13} in a mode similar to that of p115 RhoGEF. Immunocytochemical analyses revealed that KIAA0380 is distributed not only in the cell body but also in the tips of neurites where cortical F-actin is reorganized. KIAA0380 could bind to constitutively active Gα_{12} and Gα_{13} mutants in the COS7 cell transfection system (44). From these observations, it is tempting to speculate that Gα_{12} and/or Gα_{13} regulates KIAA0380 function in vivo. We found no further activation of VH-Rho-kinase by Gα_{12}/Gα_{13}, since KIAA0380 is constitutively active in transfected cells. We assumed that endogenous KIAA0380 is regulated by an unidentified endogenous regulator(s) and the inhibitor is not sufficient when KIAA0380 is over-expressed.

We considered the possible involvement of KIAA0380 in LPA-dependent neurite contraction of neuronal Neuro2a cells. Our hypothesis that KIAA0380 functions as a RhoGEF in neurite retraction was supported by findings that the N terminal fragment of KIAA0380 (KIAA0380-N) could block the neurite retraction induced by LPA. The inhibition of neurite retraction by KIAA0380-N was only partial. One explanation for the results is that the expression level of the KIAA0380-N fragment is inadequate to induce complete inhibition of neurite retraction while a high expression appeared to cause cytotoxicity.

We designed a detection system for the Rho-kinase activation state, and this system was found to be applicable to detect RhoGEFs which activate the Rho/Rho-kinase-dependent signaling pathway. Using the detection system, we found that KIAA0380 is a Rho/Rho-kinase activator in vivo. We then assessed the effects of various KIAA0380 mutants on actin reorganization and morphological changes of various cell lines, including Swiss 3T3 and Neuro2a cells. Thereafter, we characterized KIAA0380 in neuronal cells since KIAA0380 is expressed mainly in the brain. As a model, we used Neuro2a cells which are a convenient system for examining Rho action since these cells undergo rapid and dramatic Rho-mediated shape changes when treated with LPA or serum. Interestingly, in neuroblastoma N1E-115 cells, other authors dissociated LPA-induced cytoskeletal contraction from stress fiber formation; Rho translocation to the cell periphery is required for LPA-stimulated contractility but not for formation of stress fibers, since stress fibers can still be induced by activated Rho which cannot bind to the cell membrane (5). However, it remained to be clarified whether Rho is activated upon LPA-
stimulation and then translocates to the cell periphery or whether Rho first translocates to the cell periphery then is activated by a localizing RhoGEF. We could dissociate actin stress fiber formation from morphological change at the RhoGEF level; KIAA0380-DH/PH is sufficient for the former phenotype whereas a proline-rich motif (aa1081-1119) adjacent to the PH domain is required for the latter. The importance of the region extending C-terminally adjacent to the PH domain for actin reorganization has been noted for a Rac-specific GEF, Tiam1, in which the extending region contains a putative coiled coil sequence of about 40 amino acids and a following region of 300 amino acids. This extending region of about 340 amino acids is required for Tiam1-induced membrane ruffling (59). Our present results also suggest another molecular mechanism of Rho activation. The subcellular localization of KIAA0380-DH/PH is cytoplasmic with a punctate pattern, while KIAA0380-DH/PH/Pro locates underneath the plasma membrane. Thus, KIAA0380-DH/PH may activate cytoplasmic Rho and induce actin stress fibers, whereas KIAA0380-DH/PH/Pro may activate Rho located in the peripheral cell membrane or its vicinity, and consequently induce cortical actin reorganization.

In another study on full cytoskeletal contraction, (but not stress fiber formation), membrane localization of Rho was required (5), although the precise intracellular location where the GTP/GDP exchange on Rho takes place was not obvious. Based on our observation that cytoskeletal contraction followed by cell rounding and stress fiber formation can be separated using different KIAA0380 mutants, we are entertaining the notion that localization of RhoGEF determines the subpopulation of activated Rho.

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Footnotes

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1. Abbreviations used in this paper: DH, Dbl homology; GEF, guanine nucleotide exchange factor; LPA, lysophosphatidic acid; PH, pleckstrin homology; RGS, regulator of G protein signaling.
Figure legends

Figure 1.

Our system for detecting the activation state of Rho-kinase.

A, schematic representation of VH-Rho-kinase. Vimentin head domain (aa 1-87) was fused to the N-terminus of Rho-kinase. VH, vimentin head domain; Cat, catalytic domain; PH, pleckstrin homology domain. Phosphorylation of Ser71 of VH-Rho-kinase in an intramolecular (left) or intermolecular (right) manner. B, detection of the activation of VH-Rho-kinase by Western blotting. Myc-tagged activated (L63) RhoA, inactive (N19) RhoA, activated (L61) Rac or activated (L61) Cdc42 were transiently expressed in COS7 cells with Myc-tagged VH-Rho-kinase. Cells were collected in Laemmli-SDS-PAGE sample buffer and then subjected to Western blotting using 9E10 or TM71. C, the phosphorylation of Ser71 of VH-Rho-kinase is in a intramolecular manner. Myc-tagged wild-type VH-Rho-kinase (WT) or inactive VH-Rho-kinase (KDTT), with or without activated Rho-kinase (CAT), was transiently expressed in COS7 cells. After culture, with or without serum for 24h, cells were collected in Laemmli-SDS-PAGE sample buffer and then subjected to Western blotting, using 9E10 or TM71.

Figure 2.

Specificity of KIAA0380 on the Rho GTP-binding protein.

A, the activation of VH-Rho-kinase by KIAA0380. Myc-tagged KIAA0380, KIAA0380 DH or p115RhoGEF was transiently expressed in COS7 cells with Myc-tagged VH-Rho-kinase. Cells were collected in Laemmli-SDS-PAGE sample buffer and then subjected to Western blotting, using TM71 or 9E10. B, specific activation of RhoA by the fragment containing DH/PH domain of KIAA0380 in vitro. RhoA, Rac, Cdc42 or RGS-DH/PH proteins were expressed as GST-fusion proteins in E. coli and affinity purified using glutathione sepharose beads. Radioactivities of GTPγS binding to RhoA, Rac or Cdc42 expressed as GST fusion protein in E. coli were measured in C, specific activation of RhoA by KIAA0380 in vivo. FLAG-tagged Rho or Rac or Cdc42 was transiently expressed with KIAA0380 in COS7 cells. After metabolic labeling with [32P]orthophosphate, cells were lysed and FLAG-tagged GTP-binding proteins was immunoprecipitated. Radioactive nucleotides bound to GTP-binding protein were eluted and resolved by TLC. The position of GDP and GTP standard is indicated.
Figure 3.
Morphological characteristics of Swiss 3T3 cells expressing KIAA0380 or p115RhoGEF.
Serum starved Swiss 3T3 cells were microinjected with pRK5-Myc vectors containing the gene of KIAA0380 or p115RhoGEF. After 3 h, the cells were fixed and we stained F-actin using rhodamin-phalloidin (A, B and C). Cells expressing KIAA0380 (B, E and F) or p115RhoGEF (C, F, I) were identified by staining the Myc-epitope. A, D and G represent a control cell. Bar, 10 µm.

Figure 4.
Determination of the functionally important domains of KIAA0380
A, properties of the KIAA0380 proteins with respect to cell rounding and stress fiber formation. Swiss 3T3 cells were transfected with pRK5-Myc vector containing the gene indicated. PDZ domain; RGS motif; DH, Dbl homology domain; PH, pleckstrin homology domain; Pro; proline rich motif. After transfection, the cells were cultured in serum-free DMEM for 18 h, fixed and then stained for F-actin, using rhodamin-phalloidin. Protein-expressing cells were identified by staining the Myc-epitope. 'Contraction' means the protein induces fully rounded shapes and 'Actin reorganization' means the protein induced stress fiber formation. Induction of the morphology in more than 80% of transfected cells was considered positive (+). B, morphological characteristics of Swiss 3T3 cells expressing various truncated mutants of KIAA0380. Swiss 3T3 cells were microinjected with pRK5-Myc vectors containing the gene indicated. After 3 hours, the cells were fixed, stained, and identified expressing the mutant of KIAA0380, using the Myc-epitope (top) and stained F-actin, using rhodamin-phalloidin (middle). C, possible model for Rho activation by KIAA0380 mutants inducing cortical actin reorganization or stress fiber formation.

Figure 5.
Morphological characteristics of MDCKII cells or Neuro2a cells expressing KIAA0380.
Serum starved MDCKII cells were microinjected with pRK5-Myc vectors containing the gene of KIAA0380. After 3 hours, the cells were fixed and F-actin stained using rhodamin-phalloidin (C and D). Cells expressing KIAA0380 were identified by staining the Myc-epitope (A and B). The x-z scan
(A, C and E) was done along a fixed position on the y-axis (B, D and F). Neuro2a cells were transfected with pRK5-Myc vectors containing the gene of KIAA0380 and cultured in serum-free DMEM for 18 h. The cells were fixed and stained F-actin, using rhodamin-phalloidin (I and J). Cells expressing KIAA0380 were identified by staining the Myc-epitope (G and H). Cells expressing KIAA0380 were captured by short exposure (H, J and L). Bar, 10 µM.

Figure 6.
Characterization of affinity-purified antibody for the C-termini of KIAA0380.
Detection of KIAA0380 or p115Rho-GEF by immunoprecipitation and Western blotting. COS7 cells transiently expressing KIAA0380 (lane 1) or Neuro2a cells (lane 2) were subjected to immunoprecipitation, using an affinity-purified antibody raised against the C-terminal fragment of KIAA0380. (aa1056-1522). Samples were separated on a 7.5% polyacrylamide gel and then subjected to Western blotting, using an anti-KIAA0380 antibody. p115RhoGEF protein were precipitated, using an anti-p115RhoGEF antibody. Lysates of COS7 cells transiently expressing p115RhoGEF (lane 3) or Neuro2a cells (lane 4) were subjected to immunoprecipitation and Western blotting, using an anti-p115RhoGEF antibody.

Figure 7.
Distribution of KIAA0380 in Neuro2a cells. Neuro2a cells were cultured on coverslips in serum-free DMEM for 24 h (A-C). After 1 µM LPA stimulation (D-R), Neuro2a cells were fixed and stained with KIAA0380, using an affinity-purified anti-KIAA0380-C antibody (A, D, G, J, M and P) and F-actin, using rhodamin-phalloidin (B, E and H) or RhoA (K), tubulin (N) or vimentin (Q), with respective antibodies. Bar, 10 µm.

Figure 8.
Inhibition of LPA-induced neurite retraction of Neuro2a by the N-terminal fragment of KIAA0380. Cells were transfected with 1.0 µg of pRK5-KIAA0380-N (aa1-592). After transfection the cells were maintained overnight in serum-free medium and were subsequently stimulated with 1 µM LPA for 3 min. Morphologies of the cell scored were as follows: round, fully contracted; flat, flattened without...
neurite extensions, or with extensions shorter than the soma diameter; neurite, flattened with neurite extensions longer than the soma diameter. Each value is the mean ± S.D. for 100-200 cells sampled from three independent experiments.
Functions of a Rho-specific guanine nucleotide-exchange factor in neurite retraction: Possible role of a proline-rich motif of KIAA0380 in localization
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