

Rho-specific binding and guanine nucleotide exchange catalysis by KIAA0380, a Dbl family member

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Abstract Several guanine nucleotide exchange factors (GEFs) for Rho-GTPases have been identified, all of them containing a Dbl homology (DH) and pleckstrin homology (PH) domain, but exhibiting different specificities to the Rho family members, Rho, Rac and Cdc42. We report here that KIAA0380, a protein with a tandem DH/PH domain, an amino-terminal PDZ domain and a regulator of G protein signalling (RGS) homology domain, is a specific GEF for RhoA, but not for Rac1 and Cdc42, as determined by GDP release, guanosine 5'-O-(3-thio)triphosphate (GTP γ S) binding and protein binding assays. When expressed in J82 cells, DH/PH domain-containing forms of KIAA0380 induced actin stress fibers, whereas expression of the RGS homology domain prevented lysophosphatidic acid (LPA)-induced stress fiber formation.

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Key words: RhoA; Guanine nucleotide exchange factor; Actin stress fiber; Lysophosphatidic acid; J82 bladder carcinoma cell

1. Introduction

Rho family GTPases, with the main members RhoA, Rac1/2 and Cdc42, play essential roles in various cellular functions, predominantly in organization of the actin cytoskeleton as well as cell growth and transformation [1]. These GTPases serve as molecular switches which are inactive in the GDP-bound state and active in the GTP-bound state. Transition from the GDP- to the GTP-bound state is catalyzed by specific guanine nucleotide exchange factors (GEFs), some of which were first identified as oncogene products [1,2].

Meanwhile, there is a large family of about 20 distinct proteins exhibiting GEF activity towards Rho-GTPases. They all contain in tandem a Dbl homology (DH) domain which is responsible for the guanine nucleotide exchange activity and a pleckstrin homology (PH) domain thought to be involved in subcellular localization of the proteins [2,3]. Nevertheless, some of these proteins exhibit GEF activity to only one specific member of the Rho-GTPase family, e.g. RhoA, while others can activate multiple Rho-GTPases with similar efficacy [2]. Recently, a region with homology to regulators of

G protein signalling (RGS) has been identified in human p115-RhoGEF and its murine homologue Lsc [4–6]. This RGS homology domain appears to be a functional site for interaction of these GEFs with heterotrimeric G proteins. In fact, it has been shown that the GTPase activities of G α_{12} and G α_{13} are enhanced by p115-RhoGEF and that on the other hand, the guanine nucleotide exchange activity of p115-RhoGEF towards RhoA is enhanced by activated G α_{13} [4,5].

The aim of the present study was to characterize a putative new member of the Rho-GEF family, namely KIAA0380, a protein containing a DH domain in tandem with a PH domain as well as an RGS homology domain at the amino-terminus [4,7]. We report here that KIAA0380 is a highly specific GEF for RhoA, but does not interact with Rac1 and Cdc42. Overexpression of KIAA0380 in human bladder carcinoma (J82) cells resulted in actin stress fiber formation, a cellular response known to be controlled by RhoA [1], whereas expression of the amino-terminal RGS homology domain prevented actin stress fiber formation induced by the G protein-coupled lysophosphatidic acid (LPA) receptor.

2. Materials and methods

2.1. Plasmids

cDNA encoding KIAA0380 (GenBank accession number AB002378) was kindly provided by Dr T. Nagase (Kazusa DNA Research Institute, Chiba, Japan). For the expression of different myc-tagged variants of KIAA0380 in mammalian cells, i.e. the near full-length protein containing the RGS, DH and PH domains (KIAA0380-RGS/DH/PH, amino acids (aa) 111–1522), the carboxy-terminal part with the DH and PH domains (KIAA0380-DH/PH, aa 637–1522) and the amino-terminus with the RGS homology domain but lacking the DH and PH domains (KIAA0380-RGS, aa 92–637), corresponding cDNA fragments were subcloned into the pCMV3 expression vector (Stratagene).

2.2. Purification of proteins

For purification of KIAA0380, a KIAA0380-DH/PH construct was prepared by subcloning the corresponding cDNA fragment into the baculovirus transfer vector pAcGHLT-A (PharMingen), allowing for expression as a glutathione *S*-transferase (GST) fusion protein. Recombinant baculovirus was obtained by co-transfection of the construct with BaculoGold DNA (PharMingen) using lipofectamine (Gibco BRL). Sf9 cells infected with recombinant baculovirus were lysed 72 h later by sonification on ice in the presence of protease inhibitors and supernatants were prepared. Thereafter, the GST fusion protein was purified by glutathione Sepharose (Pharmacia) affinity chromatography. The GST fusion products of RhoA, Rac1 and Cdc42 were expressed in *Escherichia coli* using the pGEX vector, kindly provided by Dr A. Hall, University College London, and purified as described in [8]. The homogeneity of the recombinant proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. GDP/GTP exchange assays

Binding of GDP and the stable GTP analogue guanosine 5'-O-

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Abbreviations: DH, Dbl homology; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; GTP γ S, guanosine 5'-O-(3-thio)triphosphate; LPA, lysophosphatidic acid; PH, pleckstrin homology; RGS, regulator of G protein signalling; TRITC, tetramethylrhodamine isothiocyanate

(3-thio)triphosphate (GTP γ S) to Rho-GTPases was determined at room temperature by the filter binding method essentially as described in [9]. In brief, for GDP binding, the recombinant GTPases were first made nucleotide-free by incubation for 5 min in EDTA (2 mM)-containing loading buffer, supplemented with 10 μ M [3 H]GDP (Amersham). Thereafter, MgCl $_2$ was added to a final concentration of 5 mM and the incubation was continued for a further 20 min. Finally, purified recombinant KIAA0380-DH/PH equilibrated for 15 min in exchange buffer containing 1 mM GTP was added and the reaction continued for the indicated periods of time. For binding of GTP γ S, unlabelled GDP (10 μ M) was used in the loading buffer instead of [3 H]GDP and the exchange mixture contained 5 μ M [35 S]GTP γ S (New England Nuclear) instead of unlabelled GTP. Bound and free nucleotides were separated by filtration through nitrocellulose filters.

2.4. Protein binding assay

Binding of KIAA0380 to Rho-GTPases was carried out essentially as described in [3]. Briefly, HEK-293 cells were transfected with myc-tagged KIAA0380-RGS/DH/PH using the calcium phosphate method and 48 h later, cells were lysed at 4°C in a buffer containing 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.2% sodium deoxycholate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin. Recombinant GST-fused GTPases (~50 μ g) immobilized on glutathione Sepharose beads were first incubated with cell lysate from untransfected cells (~700 μ g protein) for 1 h at 4°C. Then, nucleotide-free, GDP-bound or GTP γ S-bound forms of the Rho-GTPases were prepared exactly as described in [3]. Thereafter, the beads were incubated for 16 h at 4°C with lysates of transfected cells (600 μ g protein). Finally, after three washes, the beads were resolved in Laemmli buffer, subjected to SDS-PAGE and transferred to nitrocellulose for Western blot analysis using an anti-myc antibody (9E10, Calbiochem). Immunoreactive proteins were visualized by enhanced chemoluminescence (Amersham).

2.5. Phalloidin staining and fluorescence microscopy

Subconfluent human bladder carcinoma (J82) cells [10] cultured as in [11] and grown in 60 mm plastic dishes were transfected with 20 μ g of DNA encoding myc-tagged KIAA0380-RGS/DH/PH, -DH/PH or -RGS using the calcium phosphate method. Twenty-four h later, cells were collected from the dishes by trypsinization and seeded for a further 24 h on culture slides (Falcon) to allow for studies of the actin cytoskeleton. For this, subconfluent monolayers of J82 cells were rinsed in Ca $^{2+}$ and Mg $^{2+}$ -containing phosphate-buffered saline (PBS), followed by incubation with and without LPA for 15 min at 37°C in PBS. Thereafter, the cells were washed twice for 5 min with PBS, fixed with 3% paraformaldehyde for 15 min and washed twice for 5 min with PBS. The cells were permeabilized by incubation for 2 min in a solution of 0.05% (v/v) Triton X-100, washed twice with PBS and then incubated for 45 min in 0.5% (w/v) fatty acid-free bovine serum albumin in PBS. Then, the cells were treated for 45 min with 5 μ g/ml mouse anti-myc antibody in 0.5% (w/v) fatty acid-free bovine serum albumin in PBS, followed by three washes with PBS. Thereafter, the cells were exposed for another 45 min to an anti-mouse IgG fluorescein isothiocyanate conjugate (1:100 dilution, Sigma) in a solution of tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (25 μ g/ml, Sigma). After three final washes with PBS, the stained cells were mounted with Moviol (Calbiochem) and examined, using a Zeiss Axiocvert microscope.

3. Results

Protein homology analysis of KIAA0380 revealed that the predicted aa sequence contains a DH domain (aa 741–928) followed by a PH domain (aa 965–1079) (Fig. 1). These sequences are 61, 64 and 48% identical to the homologous regions of murine Lsc [12], its human homologue p115-RhoGEF [13] and Lfc [14], respectively. In addition to the tandem DH/PH domains, KIAA0380 contains a PDZ domain at the amino-terminus (aa 47–111) as well as two proline-rich domains (aa 149–160 and 1089–1099, respectively). Finally, similar to Lsc and p115-RhoGEF, KIAA0380 contains near

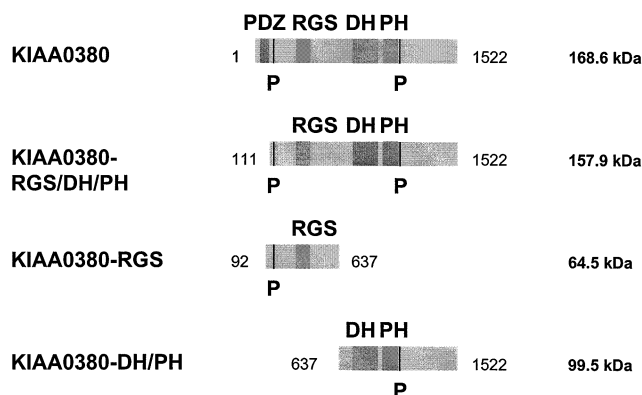


Fig. 1. Schematic representation of KIAA0380 and its domain structure. In addition to PDZ, RGS, DH and PH domains, KIAA0380 contains two proline-rich regions (P). Below the full-length protein, the various KIAA0380 variants used in this study are shown, with the corresponding aa numbers and calculated molecular weights.

the amino-terminus an RGS homology domain (aa 310–394). Thus, the sequence analysis predicted that KIAA0380 is a GEF for Rho-GTPases and it may interact with heterotrimeric G proteins and G protein-coupled receptors.

To determine potential GEF activity of KIAA0380 towards Rho-GTPases, we expressed and purified a GST-fused form of KIAA0380 (KIAA0380-DH/PH) that included the DH and PH domains as well as some additional flanking sequence. Since the amino-terminus containing the RGS homology domain may act as an intramolecular inhibitor of GEF function, as described for p115-RhoGEF [4,5], this part was deleted in the GST fusion protein. Fig. 2A shows that purified GST-KIAA0380-DH/PH potentially stimulates release of [3 H]GDP bound to purified GST-RhoA. Half-maximal release of [3 H]GDP from RhoA (0.6 μ M) was observed at 0.13 μ M KIAA0380. In contrast, GST-KIAA0380-DH/PH exhibited no GEF activity towards GST-Rac1 and GST-Cdc42. In the absence of KIAA0380, there was no release of [3 H]GDP from RhoA over the time course studied (up to 15 min) (Fig. 2B). Addition of GST-KIAA0380-DH/PH caused a marked acceleration of GDP dissociation, reaching half-maximal release at about 5 min. A similar acceleration of the exchange reaction by GST-KIAA0380-DH/PH was observed when instead of [3 H]GDP release, binding of [35 S]GTP γ S to RhoA was examined (Fig. 2C). Together, these data indicate that KIAA0380 is a potent and specific GEF for RhoA.

Some members of the Dbl family, e.g. Ect-2, bind to Rho-GTPases but do not induce the GDP/GTP exchange reaction [15], suggesting that in some cases, the DH domain serves as a binding motif, perhaps for recruitment and subcellular translocation of the GTPases. Therefore, we studied the binding specificities of KIAA0380 towards RhoA, Rac1 and Cdc42 as well as the nucleotide dependence of this interaction. For this, myc-tagged KIAA0380-RGS/DH/PH was expressed in HEK-293 cells and after cell lysis binding of the expressed protein to GST-RhoA, -Rac1 and -Cdc42, each, either in the nucleotide-free, GDP-bound or GTP γ S-bound state, was examined. As shown in Fig. 3, KIAA0380-RGS/PH/DH expressed in HEK-293 cells (cell lysate) did not bind to GST and also not to GST-Rac1 and GST-Cdc42 in any of the nucleotide binding states. In contrast, KIAA0380-RGS/PH/DH bound to GST-RhoA and this binding occurred best when RhoA was in the

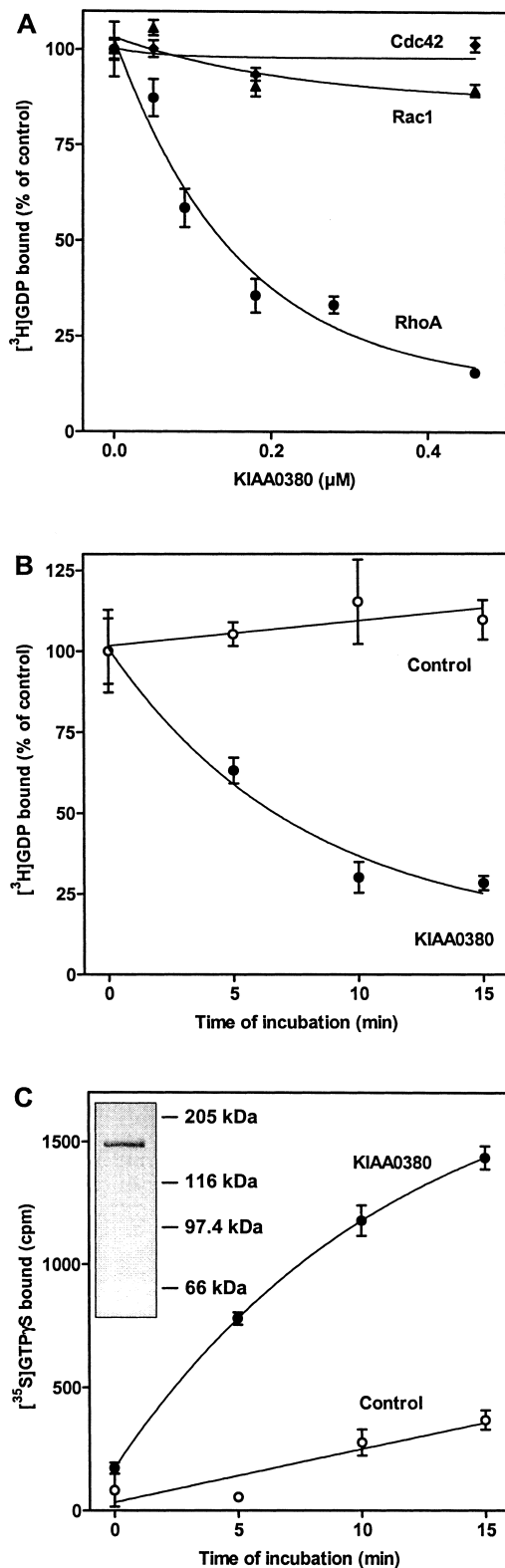


Fig. 2. RhoA-specific GEF activity of KIAA0380. A: Concentration response curve of purified recombinant GST-KIAA0380-DH/PH on release of [3 H]GDP from recombinant GST-RhoA, Rac1 and Cdc42 (0.6 μ M each), measured for 30 min. B: Time course of [3 H]GDP release from GST-RhoA (0.6 μ M) in the absence and presence of GST-KIAA0380-DH/PH (0.46 μ M). C: Time course of [35 S]GTP γ S binding to GST-RhoA (0.6 μ M) in the absence and presence of GST-KIAA0380-DH/PH (0.46 μ M). Inset: Coomassie blue-stained SDS-PAGE of purified GST-KIAA0380-DH/PH, with molecular weight markers on the right. Shown are mean \pm S.D. of a representative experiment out of 2–4, each performed in quadruplicate.

To investigate whether KIAA0380 may act as a Rho-GEF *in vivo*, several myc epitope-tagged variants of KIAA0380 were transiently expressed in J82 bladder carcinoma cells. Specifically, KIAA0380-DH/PH and KIAA0380-RGS/DH/PH, shown above to exhibit GEF and binding activity towards RhoA, respectively, as well as a variant containing the RGS homology domain, but lacking the DH and PH domains (KIAA0380-RGS, see Fig. 1), were studied. Expression of the KIAA0380 variants was verified by Western blots with an anti-myc antibody (data not shown) and by fluorescence microscopy using the same antibody. The actin cytoskeleton was visualized by labelling with TRITC-conjugated phalloidin. In cells expressing KIAA0380-RGS/DH/PH or -DH/PH, identified by positive staining with the anti-myc antibody, formation of actin stress fibers was observed (Fig. 4), similar as in cells expressing V14RhoA, a constitutively active RhoA mutant (data not shown). These results are in agreement with the notion that actin stress fiber formation is a RhoA-regulated cellular response [1] and that the KIAA0380 variants expressed in J82 cells specifically bind to and activate RhoA *in vitro* (see Figs. 2 and 3).

Since KIAA0380 contains a RGS homology domain, it may interact with heterotrimeric G proteins, as reported for p115-RhoGEF [4,5]. Thus, we finally studied the effect of KIAA0380's RGS domain on actin stress fiber formation induced by LPA, a G protein-coupled receptor agonist. As shown in Fig. 5, expression of KIAA0380-RGS by itself had no effect on the actin cytoskeleton in J82 cells, but completely prevented formation of actin stress fibers in response to LPA. Thus, DH/PH domain-containing variants of KIAA0380 can apparently activate RhoA in intact cells, whereas KIAA0380-RGS seems to function as a dominant negative mutant, probably by interfering with activation of endogenous RhoA.

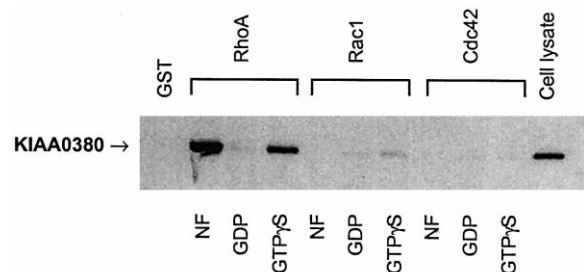
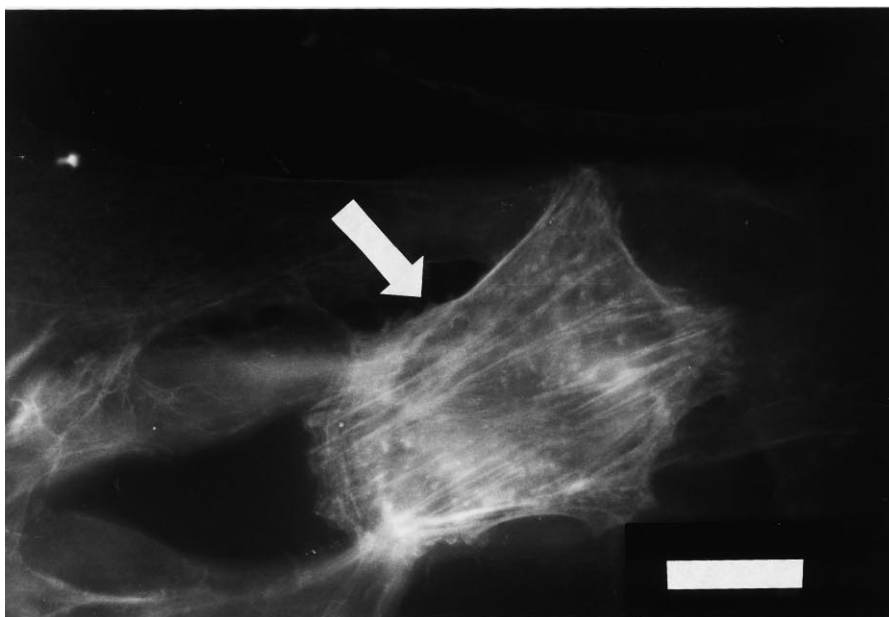


Fig. 3. RhoA-specific binding of KIAA0380. Binding of myc-tagged KIAA0380-RGS/DH/PH expressed in HEK-293 cells to GST or GST fusion proteins of RhoA, Rac1 and Cdc42 was determined as described in Section 2. The GTPases were either nucleotide-free (NF) or were binding GDP or GTP γ S as indicated. Bound KIAA0380 was detected with an anti-myc antibody.

nucleotide-free state. While binding to GDP-RhoA was not detected, there was substantial binding of KIAA0380-RGS/PH/DH to GTP γ S-liganded RhoA, although clearly less than to the nucleotide-free GTPase. Thus, KIAA0380 is not only a RhoA-specific GEF but also binds selectively only to RhoA but not to Rac1 and Cdc42.

A anti-myc antibody detection



B anti-myc antibody detection

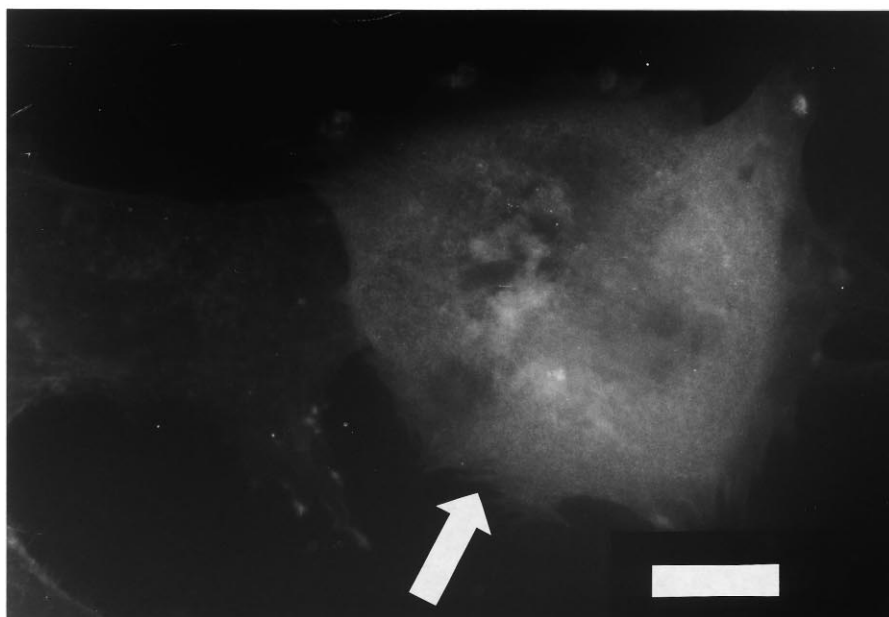


Fig. 4. Formation of actin stress fibers by KIAA0380 in J82 cells. TRITC-phalloidin staining of actin (left) and anti-myc antibody immunofluorescence (right) of J82 cells expressing (see arrows) either myc-tagged KIAA0380-RGS/DH/PH (A) or KIAA0380-DH/PH (B). Bar, 20 μ m.

4. Discussion

Alignments of KIAA0380's protein sequence with members of the Dbl family of proteins indicated that KIAA0380 contains a DH domain in tandem with a PH domain as found in all GEFs of Rho-GTPases and with specific high sequence homologies to the Rho-specific GEFs, Lsc, p115-RhoGEF

and Lfc. These data suggested that KIAA0380 is a GEF for Rho-GTPases and possibly specific for Rho. Here, we demonstrate with purified KIAA0380 and the Rho-GTPases, RhoA, Rac1 and Cdc42, that KIAA0380 is a specific and efficient GEF for RhoA, without affecting GDP/GTP exchange of Rac1 and Cdc42. Furthermore, it is shown that KIAA0380 binds specifically to RhoA, but not to Rac1 and

Cdc42. Finally, expression of KIAA0380 in J82 cells was found to induce actin stress fiber formation, a cellular response known to be under specific control by RhoA [1]. Thus, both in vitro and intact cell data indicate that KIAA0380 is a novel Rho-specific GEF.

During our studies on KIAA0380, Fukuhara et al. [16] reported that KIAA0380, termed PDZ-RhoGEF by these authors because of KIAA0380's amino-terminal PDZ domain with a so far unknown specific function, is widely expressed. In line with our direct in vitro data, KIAA0380/PDZ-RhoGEF overexpressed in NIH-3T3 or COS-7 cells was found to stimulate a Rho-specific pathway (serum response element activation), but failed to activate a pathway (activation of c-Jun N-terminal kinase) regulated by Rac and Cdc42 [16]. In intact cells, however, it has to be considered that Rho, Rac and Cdc42 can exhibit extensive overlap as well as crosstalk in regulating signalling pathways [1] and that cellular responses induced by individual Rho-GEFs are dependent on and thus limited by the levels of endogenous Rho-GTPases [17].

In line with the classic GEF-GTPase interaction model, involving stabilization of a nucleotide-free transition state of the GTPase by the GEF [3], binding of KIAA0380 to RhoA was preferential to the nucleotide-free GTPase. While binding to RhoA-GDP was not detected, there was, however, also substantial binding of KIAA0380 to GTP γ S-liganded RhoA. In comparison, Lfc, Lsc and Lbc, three other Rho-specific GEFs, were reported to bind to nucleotide-free RhoA but not to the GDP- or GTP γ S-bound states of RhoA [18]. On the other hand, mNET1, also a RhoA-specific GEF, bound GDP- and GTP γ S-liganded RhoA equally well ([19], binding of mNET1 to nucleotide-free RhoA was not reported). Finally, GEF-H1, a GEF for Rho and Rac, bound to RhoA and Rac1 irrespective of the nucleotide binding state of these

GTPases [20]. Thus, the nucleotide binding state-specificity of the interaction of Rho-GTPases with their GEFs is apparently quite distinct, even in the subfamily of Rho-specific GEFs. As KIAA0380 binds specifically to GTP γ S-liganded RhoA, it may be speculated that KIAA0380 not only acts as a GEF for RhoA, but at the same time is an effector of activated RhoA or is a carrier for the active GTPase to potential effector target sites in cells.

Like Lsc and p115-RhoGEF, KIAA0380 contains an amino-terminal RGS homology domain, suggesting that KIAA0380 may interact via this domain with heterotrimeric G proteins, as reported for p115-RhoGEF [4,5], and may thus interfere with signalling by G protein-coupled receptors. In fact, Fukuhara et al. [16] reported that KIAA0380/PDZ-RhoGEF can form via its RGS domain stable complexes specifically with activated G α_{12} and G α_{13} and that a DH/PH deletion mutant of KIAA0380/PDZ-RhoGEF reduces Rho-mediated serum response element activation by G α_{12} and G α_{13} . To study whether KIAA0380 via its RGS domain may interfere with signalling by G protein-coupled receptors, the effect of KIAA0380 on actin stress fiber formation was studied in J82 cells. This response is induced in J82 cells by several G protein-coupled receptors, including the LPA receptor, and is a Rho-mediated event [21]. While DH/PH-containing variants of KIAA0380 induced actin stress fiber formation, KIAA0380-RGS lacking the DH/PH domains by itself had no effect. However, the stimulatory effect of LPA was fully prevented by expression of the isolated RGS domain. As the related Rho-specific GEF, p115-RhoGEF, acts as a GTPase-activating protein for G α_{12} and G α_{13} [4], it may be speculated that the RGS domain of KIAA0380 shown to interact with G α_{12} and G α_{13} [16] may exhibit a similar activity towards these G proteins and thereby interrupts signalling

TRITC-phalloidin staining

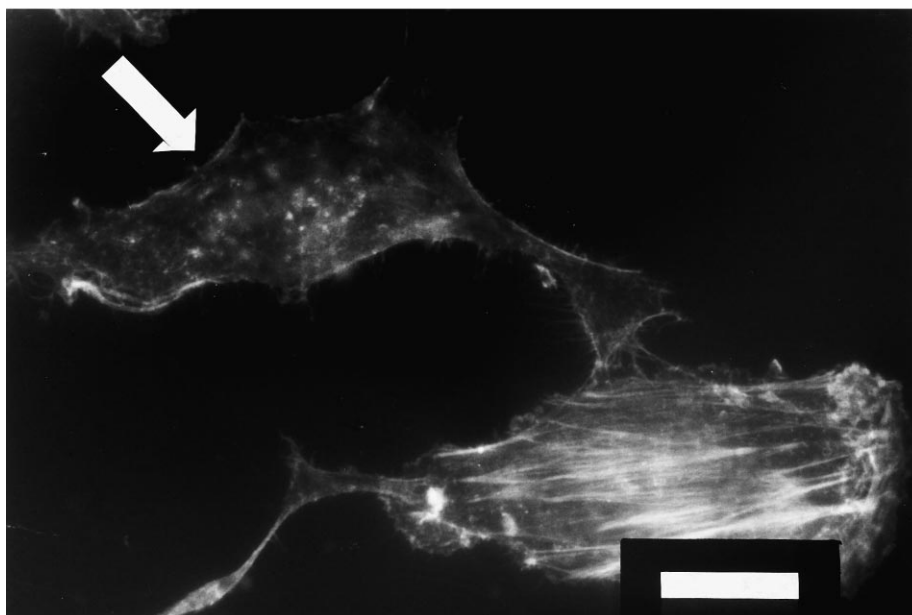


Fig. 5. Block of LPA-induced stress fiber formation by the RGS homology domain of KIAA0380. TRITC-phalloidin staining of actin (upper panel) and anti-myc antibody immunofluorescence (lower panel) of J82 cells expressing (see arrow) or not myc-tagged KIAA0380-RGS and stimulated for 15 min with 10 μ M LPA. Bar, 20 μ m.

of the LPA receptor to endogenous Rho. It remains to be studied whether KIAA0380 which is endogenously expressed in J82 cells (data not shown) mediates this LPA receptor signalling and, if yes, by which mechanisms KIAA0380 is activated, finally leading to activation of RhoA. Interestingly, the tissue distribution of the two closely related Rho-specific GEFs, KIAA0380 and p115-RhoGEF, appears to be quite different. While expression of KIAA0380 is highest in heart, brain, placenta, testis and ovary, transcripts for p115-RhoGEF were hardly detected in these tissues, whereas strong expression of p115-RhoGEF was found in spleen, thymus and peripheral blood leukocytes where less or no expression of KIAA0380 was found [13,16]. Thus, although functional differences between these two Rho-specific GEFs may exist, these two GEFs may function in Rho activation in a tissue-specific manner.

In summary, we demonstrate here that KIAA0380 specifically interacts with RhoA and efficiently stimulates GDP/GTP exchange of this GTPase, without affecting guanine nucleotide exchange of Rac1 and Cdc42 or interacting with these GTPases. In agreement, KIAA0380 induces Rho-specific pathways in intact cells. By its RGS domain, KIAA0380 may specifically link G protein-coupled receptors to Rho activation.

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