Dissociation of LPA-induced cytoskeletal contraction from stress fiber formation by differential localization of RhoA

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SUMMARY

Addition of lysophosphatidic acid (LPA) to serum-deprived N1E-115 neuronal cells results in rapid f-actin assembly accompanied by neurite retraction and rounding of the cell body due to contraction of the cortical actin cytoskeleton. LPA action is mimicked by activated RhoA, while it is blocked by dominant-negative RhoA (N19RhoA) and the Rho-inactivating C3 toxin. Using immunofluorescence analysis and high speed centrifugation we show that activated RhoA is localized to the plasma membrane. Wild-type RhoA and N19RhoA, however, are mainly cytosolic. We find that LPA-induced shape changes are preceded by translocation of RhoA from the cytosol to the cell periphery. LPA also stimulates translocation of inactive N19RhoA in the absence of ensuing shape changes. When membrane localization of RhoA is prevented by lovastatin, an inhibitor of protein isoprenylation, or by CAAX motif mutation, cytoskeletal contraction is blocked. However, the assembly of f-actin into stress fibers is not affected under these conditions. The effects of both LPA and activated RhoA are blocked by tyrosine kinase inhibitors (herbimycin, genistein, tyrphostin), but not by dominant-negative Src. We conclude that: (1) LPA-induced cytoskeletal contraction, but not stress fiber formation, requires translocation of RhoA from the cytosol to the plasma membrane; (2) translocation of RhoA occurs independently of its activation; and (3), a non-Src tyrosine kinase is involved in RhoA-stimulated contractility.

Key words: Rho, LPA, Neuronal cell, Contraction, Translocation, Actin, Cytoskeleton

INTRODUCTION

The small GTP-binding protein Rho mediates cytoskeletal remodeling and cellular shape changes in response to the serum mitogen lysosphosphatidic acid (LPA) and other extracellular agonists (for review see Machesky and Hall, 1996; Ridley, 1996; Symons, 1996). Agonist stimulation is thought to trigger the conversion of inactive Rho-GDP into active Rho-GTP, which activates specific effector pathways. In particular, activated RhoA stimulates formation of actin stress fibers and focal adhesions, cytoskeletal contraction, cell rounding and neurite retraction (Ridley and Hall, 1992; Gebbink et al., 1997). While little is still known about how Rho is activated in vivo, much has been learned about Rho downstream signaling, as various new targets of Rho have recently been isolated and characterized (for review see Machesky and Hall, 1996; Ridley, 1996). Of direct relevance to Rho-stimulated contractility is the identification and characterization of a Rho-associated protein kinase that phosphorylates and thereby inhibits myosin light chain phosphatase (Kimura et al., 1996). As a result, the phosphorylated form of myosin light chain accumulates leading to contraction of the actomyosin-based cytoskeleton. An increase in actomyosin-driven contractility underlies LPA-induced neurite retraction and cell rounding in neuronal cells (Jalink et al., 1994), and is thought to be essential for stress fiber formation in fibroblasts (Chrzanowska-Wodnicka and Burridge, 1996). In addition, there is evidence that Rho action requires tyrosine kinase activity, a notion largely based on the use of pharmacological inhibitors (Jalink et al., 1993; Nobes et al., 1995; Ridley and Hall, 1994). Interestingly, Rho-mediated shape changes in neuronal cells are accompanied by activation of the Src protein tyrosine kinase (Jalink et al., 1993, 1994), although it is not clear whether Src and Rho act in the same signaling pathway. In addition, activated RhoA stimulates protein tyrosine phosphorylation in 3T3 fibroblasts (Flinn and Ridley, 1996).

Proteins that regulate Rho activity have also been identified and characterized in recent years. These include Rho-activating GDP/GTP exchange factors, and guanine nucleotide dissociation inhibitors (GDIs), which keep Rho GTPases localized mainly in a cytosolic, inactive form (Feig, 1994; Glaven et al., 1996; Hancock and Hall, 1993; Quilliam et al., 1995). Unlike the Ras proteins, which constitutively localize to the plasma membrane, Rho proteins are mainly cytosolic (in their inactive, GDP-bound forms) and have been proposed to cycle on and off the plasma membrane (Adamson et al., 1992a,b). Studies in cell-free systems suggest that translocation of Rho GTPases from the cytosol to the membrane fraction is controlled by their activation state (Bokoch et al., 1994;
Fleming et al., 1996), but whether this notion also holds for Rho translocation in intact cells is unknown.

The present study was undertaken to analyze the subcellular distribution of RhoA in relation to its activation state and in relation to its effects on the actin cytoskeleton in NIE-115 neuronal cells. These cells provide a unique system for examining Rho action, as they undergo rapid and dramatic Rho-mediated shape changes (cell rounding and neurite retraction) when treated with LPA or other G protein-coupled receptor agonists, notably thrombin and sphingosine-1-phosphate (Jalink and Moolenaar, 1992; Jalink et al., 1993, 1994; Postma et al., 1996). We used various mutant forms of RhoA, including constitutively active and dominant-negative versions as well as a RhoA mutant that cannot be isoprenylated (C190R mutation; Gebbink et al., 1997) and studied their effects on the actin cytoskeleton. Furthermore, we have examined the involvement of protein tyrosine kinase activity, including that of Src, in RhoA-stimulated cell rounding and neurite retraction. We find that LPA-induced cytoskeletal contraction, but not the assembly of f-actin into stress fibres, requires translocation of RhoA from the cytosol to the plasma membrane. Furthermore, we show that translocation of RhoA is independent of its activation state. Finally, we present evidence that a non-Src tyrosine kinase acts downstream of RhoA to participate in cytoskeletal contraction.

MATERIALS AND METHODS

Cell culture and transfections
Mouse N1E-115 neuroblastoma cells were routinely grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 8% fetal calf serum (FCS) and antibiotics. The various cDNA constructs were cloned in pcDNA3 (Invitrogen) as described (Gebbink et al., 1997). Transient transfections were performed by exposing the cells for 14-16 hours to calcium phosphate/DNA precipitates as described (Kranenburg et al., 1995; Gebbink et al., 1997). After transfection, the cells were washed three times in PBS and received fresh medium.

Immunofluorescence and confocal microscopy
Cells were grown and transected on gelatin-coated glass coverslips. The cells were fixed for 5 minutes in 3.7% formaldehyde in PBS, and were subsequently permeabilized for 10 minutes in PBS containing 0.1% Triton X-100. Fixed cells were then blocked for 30 minutes in PBS containing 1% BSA. After the first antibody (9E10; mouse monoclonal anti-myc) incubation the cells were incubated with a combination of the second antibody (rabbit-anti-mouse-FITC, DAKO) and rhodamine-conjugated phalloidin (Molecular Probes), to detect the transfected proteins and filamentous actin in green and red, respectively. The samples were analysed on a Bio-Rad confocal microscope (MRC-600).

Morphology assays
NIE-115 cells were co-transfected with pCMV-lacZ and pcDNA3 expression plasmids encoding the indicated proteins, in a 1:3 ratio. After transfection cells were washed and received fresh medium. After 24 hours the cells were fixed in 3.7% formaldehyde. Transfected (β-galactosidase-expressing) cells were then identified using the standard X-gal conversion assay. Three different morphologies can be distinguished: (1) fully rounded (‘contracted’); (2) flattened without neurite extensions, or with extensions shorter than the soma diameter; and (3), flattened with neurite extensions longer than the soma diameter. All transfections were performed at least twice and four separate dishes were transfected per experiment. Three dishes were used to assay the morphology of 100-200 cells/dish, without prior knowledge of the dishes’ identities. The fourth dish was used to determine correct protein expression by western blotting.

Membrane preparation
Cells were washed in PBS and were harvested in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA supplemented with protease and phosphatase inhibitors. The cell suspensions were then sonicated twice for 10 seconds and the membranes were pelleted by high speed centrifugation (60,000 rpm, 1 hour). Pellet and sup fractions were dissolved in sample buffer and were analysed by western blotting.

Western blotting
Samples were run out on SDS-polyacrylamide gels and were blotted onto nitrocellulose (Schleicher and Schuell). The filters were blocked in 1% BSA. First antibodies were mouse monoclonal 9E10 (anti-myc tag) and mouse monoclonal Ab-1 (anti-src, Oncogene Science). The second antibody was horseradish peroxidase-conjugated rabbit-anti-mouse IgG (DAKO). Signals were visualized using the Amersham ECL detection kit.

RESULTS

RhoA regulation of the actin cytoskeleton in neuronal cells
In serum-free medium, N1E-115 cells adopt a flattened morphology and begin to extend neurites (Jalink and Moolenaar, 1992; Jalink et al., 1993). Serum-deprived cells show relatively modest f-actin staining in the cell periphery and at the leading edges of the growth cones of developing neurites (Jalink and Moolenaar, 1992; Jalink et al., 1993; see also Fig. 6A, upper panel). When these cells are treated with LPA or serum (which contains LPA; Moolenaar, 1995), actin is rapidly polymerized into a spherical shell just underneath the plasma membrane of the cell body, with stress fibre-like structures extending into the retracting parts of the cell (not shown, see also Fig. 3 and Jalink et al., 1993). Rapidly thereafter, the cells become fully rounded (‘contracted’) showing a spherical shell of cortical f-actin but no detectable stress fibers.

We examined how expression of wtRhoA and constitutively active RhoA (V14 and L63 mutants) affects f-actin organization in serum-deprived N1E-115 cells. Fig. 1 (upper panel) shows that wtRhoA has no significant effect on cell shape nor on f-actin distribution (although highly overexpressed RhoA occasionally triggered stress fiber formation; not shown). Expression of activated RhoA causes dramatic cell rounding with the ‘contracted’ cells showing a cortical f-actin shell (middle panel), as is observed in LPA-stimulated cells. Fig. 1 also shows that the localization of L63RhoA is strikingly different from that of wtRhoA. While wtRhoA is cytoplasmic, L63RhoA is mainly concentrated at the cell periphery, presumably at the plasma membrane, where it appears to colocalize with the cortical f-actin shell (middle panel) just underneath the plasma membrane. Biochemical analysis (high speed centrifugation) confirms that L63RhoA is localized to the membrane (see below). We also examined the effects and localization of dominant-negative N19RhoA, which is presumed to be mainly in the inactive GDP-bound form. The lower panel of Fig. 1 shows that N19RhoA, like
wtRhoA, is cytoplasmic. However, unlike wtRhoA, N19RhoA induces cell flattening and neurite extension in serum/LPA-containing medium, very similar to what is observed with the Rho-inactivating C3 toxin (Jalink et al., 1994). Although not clearly seen in Fig. 1, N19RhoA-expressing cells do show peripheral actin staining (visible after longer exposure; not shown).

Taken together, these data show that RhoA activation is necessary and sufficient to trigger the formation of a contractile shell of cortical f-actin with consequent induction of cell rounding and neurite retraction.

**Translocation of RhoA to the plasma membrane in response to LPA**

We next examined whether activation of wtRhoA by the physiological stimulus LPA would affect its localization. N1E-115 cells were transiently transfected with myc-tagged wtRhoA and were then serum-starved for 18 hours. As shown in Fig. 2 (upper and middle panel), addition of LPA triggers dramatic translocation of wtRhoA from the cytoplasm to the cell periphery, as is observed with constitutively active RhoA (compare the middle panels of Figs 1 and 2).

These results show that activated RhoA, induced either through mutation or receptor stimulation, undergoes translocation from the cytosol to the plasma membrane. This raises the question of whether activation of RhoA is required for translocation. In other words, to what extent is the translocation of RhoA controlled by its activation state? We addressed this issue by analysing the localization of constitutively inactive, GDP-bound N19RhoA before and after LPA stimulation. Fig. 2 (lower panel) shows that N19RhoA does undergo translocation in response to LPA, similar to what is observed with wtRhoA. However, whereas wtRhoA-transfected cells round up through formation of a contractile f-actin shell, the N19RhoA-transfected cells remain flattened with an unaltered f-actin distribution after LPA treatment. These results suggest that N19RhoA acts at the plasma membrane to inactivate endogenous RhoA, thereby preventing the formation of a
Fig. 2. Translocation of wtRhoA and N19RhoA in response to LPA. (A) Cells on glass coverslips were transfected with myc-wtRhoA or myc-N19RhoA. After transfection the cells were grown overnight in serum-free medium and were subsequently stimulated with LPA (1 μM) for 2 minutes. Monoclonal 9E10 was used to detect myc-Rho (left panels) and rhodamine-conjugated phalloidin to detect f-actin (middle panels). Merges are shown in the right panels. The upper panel shows that wtRhoA is predominantly cytoplasmic in serum-starved cells and does not induce contraction. After LPA stimulation, both wtRhoA (middle panels) and N19RhoA (lower panels) translocate to the plasma membrane, where apparent co-localization with f-actin is detected. However, whereas wtRhoA-transfected cells are in a fully contracted state with a cortical shell of f-actin, the N19RhoA-transfected cells stay flattened with a relaxed cytoskeleton. Note that all non-transfected surrounding cells are fully contracted. (B) Interference with LPA-induced neuronal cell rounding by N19RhoA. Cells were transfected with pCMV-lacZ and either a control vector or pCMV-N19RhoA. After transfection the cells were maintained in serum-free medium overnight and were subsequently stimulated with LPA (1 mM, 5 minutes), or were left untreated. Morphologies were then assayed as described in the Materials and Methods section. The data show that N19RhoA abrogates LPA-induced neuronal cell rounding.
cortical f-actin shell. Moreover, they reveal that activation of RhoA is not necessary for its translocation.

**LPA-induced stress fiber formation**

We observed that LPA-treated cells, before having reached the fully contracted state, show stress fibre-like structures extending from the cortical f-actin shell into the retracting parts of the cell. To study the subcellular localization of RhoA in relation to these two distinct f-actin structures, we made 3-D analyses of LPA-stimulated cells expressing myc-tagged wtRhoA. Fig. 3 shows three selected images from a complete 3-D scan representing horizontal (x/y) sections of the top, middle and bottom plane of the cell. A vertical cross-section (x/z plane) image is also shown. We find that RhoA is present along the entire plasma membrane (left four panels), except for the basement membrane. As in Fig. 2, we find apparent colocalization of RhoA with the cortical f-actin shell just underneath the plasma membrane (right four panels). Furthermore, relatively large amounts of f-actin are found at sites where the cytoplasm is retracting (x/z-scan, middle panel). From this f-actin ‘deposit’ stress fibres extend into the retracting part of the cell and are mainly found at the bottom of the cell (Fig. 3, xy bottom). We did not observe co-localization of RhoA with these stress fibres.

**Dissociation of RhoA-induced cell rounding from stress fibre formation by inhibiting protein isoprenylation**

Rho proteins are post-translationally modified at the C terminus by isoprenylation (geranyl-geranylation) of residue cysteine-190. This modification is necessary for Rho to translocate to membranes during cell activation and to interact with regulatory proteins such as GDIs (for review see Narumiya and Morii, 1993).

Treatment of N1E-115 cells with lovastatin, an inhibitor of acetylCoA reductase and hence protein isoprenylation, induces
cell flattening and neurite extension in the presence of serum, which is accompanied by dissolution of the cortical f-actin shell (Fig. 4A). Moreover, lovastatin renders the cells fully resistant to the contractile action of LPA (assayed under serum-free conditions; Fig. 4A). As shown in Fig. 4B, lovastatin also induces cell flattening (though not neurite outgrowth) in RhoL63-expressing cells maintained in serum-free medium. These results indicate that RhoA isoprenylation is essential for full cytoskeletal contraction and cell rounding. Most likely, isoprenylation serves to target RhoA to the plasma membrane. An additional or alternative possibility is that isoprenylation of RhoA is required for its interaction with a specific target protein.

When RhoL63-expressing cells are exposed to lovastatin, the protein is found mainly in the cytoplasm (Fig. 4C, left panel). We found that RhoL63 is still capable of promoting the assembly of f-actin into stress fibre-like structures under these conditions (Fig. 4C, middle panel), whereas cell rounding is abolished (Fig. 4B). Thus, membrane localization of activated RhoA is essential for full cytoskeletal contraction, but is not required for stress fiber formation (and hence initial contraction).

In addition to using lovastatin, we used a CAAX motif mutant of L63RhoA (C190R mutation), that cannot be isoprenylated and therefore remains in the cytoplasm (Gebbink et al., 1997). We expressed L63/R190RhoA or L63RhoA in N1E-
2423 RhoA localization and cytoskeletal remodeling

115 cells, prepared crude membranes, and analyzed the distribution of both Rho proteins by western blotting (membrane versus cytoplasmic fractions). Fig. 5A shows that RhoL63 is found exclusively in the membrane preparation, in accordance with the confocal data (Fig. 1, middle panel). Moreover, when cells were treated with genistein, which induces cell flattening and neurite extension (see below), the localization of L63RhoA was not affected, suggesting that tyrosine kinase activity is not required for targeting active RhoA to the plasma membrane (Fig. 5A). The C190R isoprenylation mutation completely abolishes membrane association, since L63/R190RhoA is found exclusively in the cytoplasmic fraction (Fig. 5A). Fig. 5B shows that the L63/R190 mutant fails to induce any shape changes (although at high expression levels L63/R190RhoA still has some rounding activity; not shown). This reinforces the results obtained with lovastatin indicating that RhoA isoprenylation, and hence membrane localization, is essential for complete cytoskeletal contraction. Of note, L63/R190RhoA is still capable of inducing stress fibres (Fig. 5C), demonstrating again that membrane localization of RhoA is required for full

Fig. 5. Rho-stimulated cytoskeletal contraction requires membrane localization. (A) Cells were transfected with expression vectors encoding myc-tagged versions of either RhoL63 or RhoL63/R190 and were either left untreated or were treated with genistein (100 μM). After 24 hours, the cells were harvested and membrane fractions were prepared by high speed centrifugation. Pellet (membrane) and sup fractions were then analyzed by western blotting using 9E10. RhoL63 is localized at the membrane also in the presence of genistein. Note that membrane localization is abolished by the C190R mutation. (B) Cells were transfected with lacZ and either a control vector or expression vectors encoding RhoL63 or RhoL63/R190. The morphology of the transfected cells was then assayed as above. The rounding activity of RhoL63 is virtually lost through mutation of residue C190. (C) Immunofluorescence analysis of RhoL63R190-expressing cells. Cells were transfected with myc-RhoL63R190 and were grown in serum-free medium overnight. It is seen that RhoL63R190 is cytoplasmic (left panel), in agreement with the above cell fractionation data. Like lovastatin-treated RhoL63, RhoL63R190 does not induce cytoskeletal contraction, although it is still capable of assembling actin into stress fibres (middle panel).
cytoskeletal contraction, but not for the initial contraction leading to stress fiber formation.

**Requirement for a non-Src tyrosine kinase acting downstream of RhoA**

We previously showed that genistein, a tyrosine kinase inhibitor, blocks LPA-induced neuronal cell rounding and neurite outgrowth (Jalink et al., 1993). In addition, we have shown that c-Src is rapidly activated in response to LPA in N1E-115 cells (Jalink et al., 1993). We therefore analyzed the role of tyrosine kinase activity, particularly that of c-Src, in RhoA signaling in further detail.

Treatment of N1E-115 cells with the tyrosine kinase inhibitor herbimycin A prevents LPA-induced neuronal shape changes in serum-deprived cells (Fig. 6A). Furthermore, herbimycin A induces cell flattening and neurite outgrowth in the presence of serum, which is accompanied by dissolution of the cortical f-actin shell (Fig. 6A). Similar effects were observed with genistein and tyrphostin 25 (results not shown). If tyrosine kinase activity only functions upstream of RhoA, L63RhoA-induced cell rounding should not be affected by herbimycin A. To test this, N1E-115 cells were transfected with RhoL63 and were subsequently serum-starved for 24 hours to induce cell flattening and neurite extension in the non-transfected cell population. When the cells were treated with herbimycinA, RhoL63-induced cell rounding was markedly inhibited (though not completely; Fig. 6B). Since membrane localization of RhoA is not affected by genistein (Fig. 5A and results not shown), it follows that a protein tyrosine kinase acts downstream of active RhoA to participate in neuronal cell rounding.

Finally, we assessed the effects of various Src proteins (wild type, constitutively active and dominant-negative) on N1E-115 cell shape. We found that expression of wt or activated c-Src counteracts rather than potentiates LPA-induced cell rounding (Fig. 7A,B). Furthermore, we did not observe any inhibitory effect of dominant-negative (kinase-dead) Src on LPA-induced cell rounding, although the protein was highly expressed (Fig. 7A,B). These results argue against c-Src driving cytoskeletal contraction in response to LPA.

**DISCUSSION**

In this study we have examined the subcellular distribution of RhoA in N1E-115 neuronal cells and how this distribution is affected following LPA stimulation. Furthermore, we have assessed the effects of various (mutant) Rho proteins on the actin cytoskeleton. N1E-115 cells represent a convenient system for examining Rho action, as these cells undergo rapid and dramatic Rho-mediated shape changes when treated with LPA or other receptor agonists such as thrombin or sphingosine-1-phosphate (Suidan et al., 1992; Tigyi and Miledi, 1992; Jalink et al., 1994; Postma et al., 1996; Tigyi et al., 1996). Shape changes are preceded by rapid f-actin polymerization, possibly as a result of Rho-mediated activation of PI-5-kinase (Ren et al., 1996; Shibasaki, 1997). We have previously shown that active RhoA mediates LPA-induced cell rounding and neurite retraction, whereas inactivation of RhoA by C3 toxin or dominant-negative N19RhoA leads to cell flattening and neurite outgrowth in the presence of serum (cf. Jalink et al., 1994; Gebbink et al., 1997; Kamata et al., 1994).

Although microscopic analysis shows that activated L63RhoA colocalizes with the contractile f-actin shell just underneath the plasma membrane, our biochemical experiments reveal that activated RhoA, but not non-isoprenylatable L63R190RhoA, RhoA localizes to membranes rather than the cytoskeleton. Since L63RhoA induces the formation of a contractile f-actin shell we conclude that activation of RhoA by...
LPA can fully account for the dramatic effects of LPA on the neuronal actin cytoskeleton. We find that both wtRhoA and inactive (GDP-bound) N19RhoA are mainly cytosolic. However, whereas over-expressed wtRhoA has little or no effect on the cytoskeleton, N19RhoA provokes the dissolution of the cortical F-actin shell in the presence of LPA or serum. It follows that RhoA activation is not only sufficient to induce cytoskeletal contraction, but also that basal RhoA activity is required for maintaining this contraction.

When activated by LPA stimulation, RhoA undergoes rapid translocation to the cell periphery. Also in MDCK cells, stimuli such as phorbol ester and hepatocyte growth factor induce translocation of wtRhoA from the cytosol to the plasma membrane (Takaishi et al., 1995). Interestingly, our findings indicate that RhoA translocation is required for LPA-stimulated contractility but not for the assembly of F-actin into stress fibers, as stress fibers are still induced by activated cytoplasmic RhoA (i.e. by L63RhoA in the presence of lovastatin or by L63R190RhoA). In addition, we detect stress fibres in LPA-treated cells that are not yet fully rounded. The location of these stress fibers (extending from the rounded cell body into the retracting part of the cell), suggests that they may contribute to cell rounding, possibly by transmitting actomyosin-based forces to distant membranes.

Full cytoskeletal contraction, but not stress fiber formation, requires membrane localization of RhoA. This suggests that the effector pathway(s) leading to full cytoskeletal contraction can only be fully activated at the plasma membrane. After LPA stimulation, an initial contractile force, which might be generated by cytoplasmically activated RhoA, triggers the formation of actin stress fibres (Chrzanska-Wodnicka and Burridge, 1996). However, this contractile force is apparently not sufficient to mediate the full cytoskeletal contraction leading to cell rounding. The different nature of the two RhoA-induced actin structures (shell vs fiber), as well as their different localization (cortex vs cytosol), suggests that their formation is regulated by different effector pathways. Since stress fiber assembly precedes the formation of the cortical shell, one could argue that shell formation is the ultimate result of extensive actin bundling, thus being a more pronounced manifestation of the same phenomenon. However, stress fiber-like structures are not observed within the cortical shell. Furthermore, stress fibers emanate from distinct sites (focal adhesions) at the basement membrane, whereas cortical shell formation is independent of cell-matrix interaction, strongly suggesting that distinct signalling pathways are involved.

An important finding is that LPA-induced translocation of RhoA in intact cells can occur independently of its activation, as inferred from our experiments using N19RhoA. At present, the nature of the signal that promotes receptor-stimulated translocation of RhoA is unknown; it could be a specific modification of the receptor or an as yet unidentified factor in the extracellular environment.
translocation of RhoA is unknown. That N19RhoA undergoes translocation in response to LPA raises the question of whether RhoA activation (i.e. GTP-loading through enhanced GDP/GTP exchange) occurs in the cytosol, at the plasma membrane, or at both locations. In vitro studies indicate that GTP/S-loaded RhoA localizes to membranes (Bokoch et al., 1994; Fleming et al., 1996), which supports our finding that L63RhoA is found exclusively at the plasma membrane. This suggests that all activated RhoA molecules undergo translocalization under physiological conditions. However, these localization studies do not reveal where the GDP/GTP exchange on RhoA takes place.

Finally, we have addressed the involvement of tyrosine kinase activity in receptor-RhoA signalling. We have previously shown that LPA-induced cell rounding and neurite retraction in N1E-115 cells is genotype-sensitive and accompanied by c-Src activation (Jalink et al., 1993). Furthermore, activated RhoA causes increased protein tyrosine phosphorylation in 3T3 fibroblasts (Flinn and Ridley, 1996). The present results, obtained with pharmacological inhibitors, dominant-negative Src and L63RhoA, argue against a role for c-Src in RhoA-stimulated contractility. The partial sensitivity of L63RhoA-induced cell rounding to tyrosine kinase inhibitors suggests that a protein tyrosine kinase acts downstream of RhoA to participate in neuronal cell rounding. Recent studies have indicated that the activation of the Rho-associated ser/thr kinase ROKα/ROkinase is sufficient to mediate stress fibre formation (Leung et al., 1996; Amano et al., 1997). Activation of ROKα/Rho-kinase leads to inactivation of myosin light chain phosphatase, in turn leading to enhanced myosin light chain phosphorylation, contractility and, finally, stress fibre formation (Kimura et al., 1996, Leung et al., 1996; Amano et al., 1997). In addition, activation of PI-3-kinase may contribute to Rho action (Ren et al., 1996; Shibasaki et al., 1997). In 3T3 cells, Rho-mediated stress fibre formation requires protein tyrosine kinase activity (Ridley and Hall, 1994). Taken together, the results support a model in which multiple RhoA-stimulated pathways cooperate to induce full cytoskeletal contraction. One of these pathways involves activation of a non-Src protein tyrosine kinase that may act in parallel with the ROKα/Rho-kinase-pathway. An alternative or additional possibility is that tyrosine kinase activity functions downstream of ROKα/Rho-kinase. Identification and characterization of this protein tyrosine kinase remains a challenge for future studies.

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