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LPA-induced mutually exclusive subcellular localization of active RhoA and Arp2 mRNA revealed by sequential FRET and FISH

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Abstract

We previously demonstrated that mRNAs for the subunits of the Arp2/3 complex localize to protrusions in fibroblasts (Mingle et al. in *J Cell Sci* 118:2425–2433, 2005). However, the signaling pathway that regulates Arp2/3 complex mRNA localization remains unknown. In this study we have identified lysophosphatidic acid (LPA) as a potent inducer of Arp2 mRNA localization to protrusions in fibroblasts via the RhoA-ROCK pathway. As RhoA is known to be activated locally in the cells, we sought to understand how spatial activation of Rho affects Arp2 mRNA localization. By sequentially performing fluorescence resonance energy transfer (FRET) and fluorescence in situ hybridization (FISH), we have visualized active RhoA and Arp2 mRNA in the same cells. Upon LPA stimulation, approximately two times more cells than those in the serum-free medium showed mutually exclusive localization of active RhoA and Arp2 mRNA. These results demonstrate the importance of localized activation of Rho in Arp2 mRNA localization and provide new insights as to how Rho regulates Arp2/3 complex mRNA localization. To our best knowledge, this is the first report in which FRET and FISH are combined to detect localized protein activity and mRNA in the same cells. This method should be easily adopted for the detection of other fluorescence protein based biosensors and DNA/RNA in the same cells.

Keywords

Arp2/3 complex; Lysophosphatidic acid; Fibroblasts and ROCK

Introduction

The Arp2/3 complex is an actin polymerization nucleator (Mullins et al. 1998), which is localized at the leading lamellae of migrating cells (Machesky et al. 1997; Welch et al. 1997). We previously demonstrated that the mRNAs for all subunits of the Arp2/3 complex localize to the leading protrusions of fibroblasts, suggesting a mechanism of targeting protein complex via local protein synthesis (Mingle et al. 2005). We also showed that Arp2 mRNA localization is dependent on external factors as serum starvation decreased the targeting of Arp2 mRNA to the protrusions while serum stimulation increased it (Mingle et al. 2005). It remained unknown

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which components in serum and what signaling pathways in the cells are important for the localization of Arp2 mRNA in fibroblasts.

Lysophosphatidic acid (LPA) is a major component of serum. It was shown to induce activation of the small GTPase RhoA, which in turn regulates many downstream effects including cytoskeleton dynamics and cell migration (Chrzanowska-Wodnicka and Burridge 1996; Ridley et al. 1999; Fukata et al. 2003; Raftopoulou and Hall 2004). Through its downstream effectors such as ROCK and diaphanous related formin proteins, Rho is known to stimulate stress fiber formation and actomyosin contraction (Ridley and Hall 1992; Kimura et al. 1996; Totsukawa et al. 2000; Watanabe et al. 1997; Tominaga et al. 2000). Conventionally, Rho was believed to be activated at the rear of migrating cells (Etienne-Manneville and Hall 2002; Worthylake et al. 2001) and to promote actomyosin-mediated contraction to release the cell adhesion from the extracellular matrix and push the cytoplasm forward (Conrad et al. 1993; Jay et al. 1995; Cheresch et al. 1999). However, Rho has also been shown to be activated at the leading edge (Kurokawa and Matsuda 2005; Kurokawa et al. 2005; Pertz et al. 2006). Such local activation of Rho has been proposed as a way to regulate cell polarity and directed migration (Goulimari et al. 2005). LPA stimulation or expression of constitutively active RhoA led to leading lamella localization of β -actin mRNA (Latham et al. 1994, 2001). However, it is not known whether RhoA activity is required for the localization of Arp2/3 complex mRNAs. Furthermore, the spatial relationship of local activation of RhoA and local mRNA enrichment in the same cells has not been reported.

Fluorescence resonance energy transfer (FRET) is a method that has been used to detect molecular interactions, conformation changes and activity states (Tsien et al. 1993; Piston and Kremers 2007). Indeed, recent information about the local activation of Rho largely came from studies using FRET (Kurokawa and Matsuda 2005; Kurokawa et al. 2005; Pertz et al. 2006). Because of its importance for cell polarity and directed cell migration, localized activation of Rho could be a key in regulating Arp2 mRNA localization. Therefore, gaining valuable insights is expected if local RhoA activity and mRNA localization could be detected in the same cells. However, detecting active Rho using FRET and an mRNA using fluorescence in situ hybridization (FISH) in the same cells has not been reported.

In this report, we have demonstrated that LPA is a potent stimulus for Arp2 mRNA localization and exerts its function through the Rho-ROCK pathway. Furthermore, we have developed a method to detect FRET and mRNA in the same cells, which reveals enhanced mutual exclusive localization of active RhoA and Arp2 mRNA in response to LPA stimulation.

Materials and methods

Reagents

Diethyl-pyrocabonate (DEPC), *Escherichia coli* tRNA, and heparin were from Sigma (St Louis, MO). Bovine serum albumin (BSA, protease and nuclease free), sheep anti-DIG (peroxidase conjugated) antibody and digoxigenin-11-dUTP (DIG-11-dUTP) were from Roche (Indianapolis, IN). Mouse anti-DIG antibody was from Jackson ImmunoResearch Laboratories (Westgrove, PA). Eagle's minimum essential medium (MEM), fetal bovine serum (FBS) and trypsin/EDTA were from Mediatech (Herndon, VA). Pathogen-free fertilized chicken eggs were purchased from Charles River SPAFAS (Franklin, CT). Tyramide signal amplification (TSA) reagents were purchased from Perkin Elmer (Boston, MA). LPA (18:1) was from Avanti Polar Lipids, Inc. (Alabaster, AL) and Y-27632 was from Calbiochem (La Jolla, CA). Other general chemicals were from Sigma and Fisher (Pittsburgh, PA).

Cell culture and transfection

Chicken embryo fibroblasts (CEFs) were prepared from day 12 chicken embryos and cultured as described (Mingle et al. 2005). The cells were plated onto gelatin-coated glass cover slips or grid glass cover slips (Bellco Biotechnology, Vineland, NJ) so that the cell density would reach 50–70% confluence the next day for transfection. Transfection was performed with Lipofectamine Plus following the manufacturer's instruction. After transfection, the cells were either cultured in normal growth medium (10% FBS in MEM with 1% penicillin and streptomycin) or in serum-free medium for 18 h before stimulated with 2 $\mu\text{g/ml}$ (or $\sim 4 \mu\text{M}$) of LPA. This dose of LPA was based on that used by Chrznowska-Wodnicka and Burridge (1996). It is slightly higher than the estimated LPA concentration of $\sim 2.8 \mu\text{M}$ in the medium containing 10% of FBS (Tokumura et al. 1994) and is within the commonly used range of higher nanomolar to the tens of micro-molar (Latham et al. 1994; Park et al. 2007; Chen et al. 2006; Kamrava et al. 2005).

Quantification of Rho activity using Rho-GTP pull-down assay

This assay was performed similarly as described previously (Ren et al. 1999). Briefly, cells were serum starved overnight and LPA stimulated for 5 or 10 min. The cells were washed with ice-cold PBS and lysed in 25 mM Tris, pH 7.5, 250 mM NaCl, 0.05% Triton X-100, 0.25% sodium deoxycholate, 0.05% SDS, and 5 mM MgCl_2 , supplemented with protease inhibitors. Cleared cell lysate was incubated at 4°C for 1 h with GST-rhotekin RBD attached to glutathione-agarose beads that were prepared according to Ren et al. (1999) using an expression plasmid (courtesy of Dr. Martin Schwartz). The beads were then washed three times with wash buffer (50 mM Tris, pH 7.5, 75 mM NaCl , 0.5% Triton X-100, and 5 mM MgCl_2) and boiled in SDS-PAGE sample buffer. The activity of Rho was determined by comparing the pull-down Rho-GTP versus the total Rho on Western blots.

Quantification of fluorescence quenching of fluorescent proteins by in situ hybridization

To assess the extent of fluorescence quenching of fluorescent proteins by FISH, CEFs were cultured on the grid cover slips then transfected with expression plasmids for fluorescent proteins eGFP, CFP, YFP or mCherry. About 24 h after transfection, the cells were fixed and imaged using an Olympus BX61 microscope with IPLab software (version 3.6.5, Scanalytics, Inc. Fairfax, VA). The cell samples were then processed by FISH. The same cells were identified based on their coordinates on the grid and imaged again with identical acquisition parameters. To quantify the fluorescence intensity of the transfected cells before and after the FISH process, the background autofluorescence was subtracted and the total fluorescence of each transfected cell was obtained. The post-FISH fluorescence intensity was divided by the prior-FISH fluorescence intensity of the same cells to generate the percentage of fluorescence retained after FISH treatment.

Quantification of mRNA and RhoA-GTP localization

Localization of mRNA was quantified visually as described previously (Mingle et al. 2005). Briefly, only the cells with >60% of the total Arp2 mRNA enriched at the leading lamellae or with >70% of total Arp2 mRNA at the leading and trailing edge combined were scored as localized. Cells that failed to meet these criteria were scored as not localized. This method was verified previously using fluorescence intensity analysis to score intracellular mRNA localization (Mingle et al. 2005). At least 300–500 cells from three independent experiments were scored for each condition with the sample identity concealed. In the cells transfected with Rho inhibitor C3 exoenzyme (C3), about 20–30% of them showed reduced spreading but with long processes. With few exception, the mRNA signal was concentrated in the cell body instead of the processes and the vast majority of these cells were scored as not localized. In contrast to the cells transfected with C3, those treated with Rho-kinase (ROCK) inhibitor Y-27632 did

not exhibit noticeable morphological change as compared to controls. For active RhoA (Rho-GTP) localization, a cell was deemed localized for Rho-GTP if its Rho-GTP was not uniformly distributed in the cytoplasm or the membrane. To quantify the spatial overlap between Rho-GTP and Arp2 mRNA, images were rescaled, rotated and aligned by aligning the CFP-YFP images combined set in as partially transparent layer to the Arp2 mRNA and anti-GFP images and applying the same transformation to the resulting FRET image. The region of localized Rho-GTP in a cell was masked using Slidebook software (Intelligent Imaging Innovations, Denver, CO). The fluorescence intensity of the Arp2 mRNA in this masked region was then calculated and divided by the total Arp2 mRNA fluorescence intensity of the whole cell. This method was chosen instead of a colocalization algorithm because the FRET image may not match exactly with the FISH image. Unless otherwise stated specifically, statistical analysis was performed using the Student's *t* test.

Sequential FRET and FISH

Cells were transfected with the Raichu-RhoA constructs on grid cover slips for 24 h and then either immediately processed or serum starved overnight and LPA stimulated for 10 min before further processing. The cells were fixed with Fixation Buffer (4% formaldehyde in PBS) for 20 min at room temperature then on ice. For FRET image acquisition, a cover slip was washed ~10 min and mounted to a custom-made device which consists of a lower and an upper ring. For mounting, the cover slip was first placed into the lower ring. The upper ring was then screwed down as such that the outer rim of the grid cover slip with fixed cells was pressed by two inter-locked metal rings to create a small open chamber. A small amount of PBS was added into the chamber to cover the cells. The device with mounted cover slip was then placed on the stage of a Zeiss LSM510 confocal microscope. Spectral scan images were acquired using a two-photon module with excitation wavelength at 820 nm. DIC images were also acquired to document the coordinates of the cells on the grid cover slip.

It is crucial to minimize the time of the cell sample in PBS before the in situ hybridization. Even though we made sure that the cells were fixed and the solutions, glass slides and cover slips were all properly treated to eliminate RNase activity, heavy loss of mRNA signal remained a problem after the FRET image acquisition when we started to develop the sequential FRET and FISH method. After a series of tests, we found that the mRNA in the fixed cells was not stable if the fixed cells were kept in PBS (DEPC treated). Specifically, after fixation (20 min, 4% formaldehyde in PBS), the Arp2 mRNA in the cells remained stable in PBS at room temperature for 2 h (~80–90% compared to immediately processed control sample) but was significantly lost (~50–60%) after 4 h in PBS at room temperature. Heavy loss of the Arp2 mRNA was also observed if the fixed cells were kept in PBS at 4°C overnight. We did find that Arp2 mRNA in the cells was stable if the samples were kept in the Fixation Buffer at 4°C (80–90% after 10 days). Therefore, we kept the fixed cell samples in the Fixation Buffer on ice until they were washed with PBS for 10 min then mounted for FRET imaging. After the FRET image acquisition which was limited to ~1.5 h, the cover slip was immediately dismounted and returned to the Fixation Buffer on ice until all the cover slips for FRET imaging were completed then processed for FISH together. These measures dramatically improved the preservation of the Arp2 mRNA (likely other RNAs as well) and we were able to acquire CFP-YFP and phase-contrast images without significant loss of the Arp2 mRNA.

The FISH process was performed as described previously (Mingle et al. 2005). Because this process quenches the fluorescence of CFP/YFP (see Fig. 5), immunostaining with anti-GFP antibodies was used to identify the transfected cells following the FISH process. After FISH and immunostaining, the cells that had been imaged for FRET were identified according to their recorded coordinates on the grid cover slip and imaged for Arp2 mRNA fluorescence.

Processing of FRET images

For the FRET images acquired using the Zeiss LSM510 confocal microscope, the specific contribution of CFP and YFP in the spectral scanning data was obtained by linear unmixing. To unmix the images, reference images were first acquired from cells transfected with only YFP or CFP. The YFP-only and CFP-only reference spectra were saved to the spectral database and used to unmix the YFP and CFP images of the Raichu-RhoA samples using the linear unmixing algorithm of LSM510Meta software. The YFP/CFP ratio was then calculated. This approach was chosen because a spectral bleed through correction may not be necessary due to our use of the Raichu-RhoA biosensor which contains equal donor and acceptor in the same molecule. To display the YFP/CFP ratio, a custom Image J plug-in was used to set the ratio values between 0.5 and 1.9 and converted the values between 1.9 and 5 to values between 2 and 255. The resulting FRET images were pseudo-colored using a custom-made lookup table for Image J.

Results

LPA stimulates Arp2 mRNA localization

We previously demonstrated that in fibroblasts Arp2/3 complex mRNAs localize to protrusions in a serum dependent manner (Mingle et al. 2005). However, the signaling pathway that regulates Arp2/3 complex mRNA localization is totally unknown. In this study, we sought to investigate this signaling pathway and chose Arp2, a core component of the complex, as the subject of study. We started by identifying stimulants in serum for Arp2 mRNA localization using CEFs as a model system. Among the tested candidates, LPA was identified as a potent inducer of Arp2 mRNA localization. Serum starvation significantly reduces the level of Arp2 mRNA localization in the CEFs (Mingle et al. 2005). Compared to the basal level of Arp2 mRNA localization in serum-starved cells, 2 μ g/ml of LPA was able to significantly increase the percentage of cells with Arp2 mRNA localization after only 10 min of stimulation, demonstrating that LPA is a potent inducer of Arp2 mRNA localization (Fig. 1).

RhoA/ROCK pathway is required for Arp2 mRNA localization

One of the best studied signaling pathways stimulated by LPA is the RhoA pathway. RhoA is a small GTPase that is involved in many aspects of cytoskeletal organization and cell migration (Chrzanowska-Wodnicka and Burridge 1996; Pertz et al. 2006). Additionally, RhoA GTPase activity is required for β -actin mRNA localization in fibroblasts (Latham et al. 2001). To test if Rho signaling is required for serum/LPA induced Arp2 mRNA localization, we first confirmed that LPA induces strong Rho activation in our cell system (Fig. 3a). We then used the C3 exoenzyme (C3), a Rho inhibitor (Jalink et al. 1994; Yamamoto et al. 1993) to treat the CEFs. The efficacy of this inhibitor on RhoA activity was confirmed in our cells by diminished stress fibers after treatment with C3 (Fig. 2a, c). As shown in Figs. 2b–d and 3b, d, Arp2 mRNA localization was dramatically inhibited in C3-transfected cells cultured in serum and those serum-starved then stimulated with LPA. These results suggest that Rho activity is necessary for Arp2 mRNA localization to the leading protrusions.

Rho-kinase (ROCK) is a downstream effector of RhoA (Totsukawa et al. 2000). ROCK can phosphorylate myosin light chain and inhibit myosin light chain phosphatase thereby activating myosin which is required for the localization of β -actin and Arp2 mRNAs (Amano et al. 1996; Kimura et al. 1996; Totsukawa et al. 2000; Latham et al. 2001; Mingle et al. 2005). To determine if ROCK activity is important for mediating Arp2 mRNA localization in response to serum or LPA, we incubated the CEFs with Y-27632, a ROCK specific inhibitor (Uehata et al. 1997). The inhibitor was able to block both serum- and LPA-induced Arp2 mRNA localization (Figs. 2e, 3c–d), suggesting that ROCK activity is necessary for Arp2 mRNA

localization. Similar inhibition of mRNA localization for Arp3 (another subunit of the Arp2/3 complex) by C3 and Y-27632 was also observed (data not shown).

Establishment of a method to detect intracellular distribution of RhoA activity and Arp2 mRNA in the same cells

Our results demonstrate that LPA stimulates while C3 and Y-27632 inhibit Arp2 mRNA localization, suggesting that activation of the Rho-ROCK pathway promotes Arp2 mRNA localization. Interestingly, we observed that global activation of RhoA by transfection of constitutively active RhoA into CEFs inhibited Arp2 mRNA localization (Mingle and Liu, unpublished). Because Rho is known to be temporally and spatially regulated (Kurokawa and Matsuda 2005; Kurokawa et al. 2005; Nakamura et al. 2005a; Pertz et al. 2006; Yoshizaki et al. 2003), which is important for cell polarity and directed cell migration (Goulimari et al. 2005), a loss of localized activity of RhoA by global activation of RhoA might be the cause of loss of Arp2 mRNA localization. In other words, not only the activity of RhoA but also where it is activated may play a key role in the distribution pattern of Arp2 mRNA in the cell. We decided to assess how the spatial pattern of active Rho correlates with that of Arp2 mRNA before and after LPA stimulation. To achieve this goal, we must be able to detect RhoA activity and Arp2 mRNA in the same cells. FRET has been successfully used to detect subcellular activation of Rho (Kurokawa et al. 2005; Nakamura et al. 2005b; Pertz et al. 2006; Yoshizaki et al. 2004) and we have used FISH to detect multiple mRNAs in the same cells (Mingle et al. 2005), so a combination of these two techniques would be an obvious choice. To visualize active Rho in the cells, we transfected the CEFs with CFP-YFP based Raichu-RhoA biosensors that were previously used to report intracellular RhoA activity (Yoshizaki et al. 2003). We confirmed that the RhoA constructs exhibited corresponding levels of FRET for wild-type (WT), constitutively active (CA) and dominant negative (DN) Rho in the CEFs (Fig. 4). After verification of the RhoA biosensor, we went on to transfect the cells with the WT Raichu RhoA construct then fixed the cells for FISH process to detect intracellular distribution of active RhoA and Arp2 mRNA. Although a combination of FISH and immunofluorescence staining has long been used by many investigators including us (Speel et al. 1994; Liu et al. 2002, 2006), the combination of FISH and fluorescence protein is less straight forward. We found that the fluorescence of CFP and YFP was significantly lost after the FISH process. To assess the impact of FISH on the fluorescent proteins, we tested several commonly used fluorescent proteins: eGFP, CFP, YFP and mCherry. As shown in Fig. 5, after FISH, there was indeed a very significant loss of the fluorescence of these proteins. Even though the quenched fluorescent protein can be detected using antibody after the FISH (Donadoni et al. 2004; Kim et al. 2007), the ratio of YFP/CFP fluorescence cannot be recovered after the FISH process. To overcome this problem, we chose to perform FRET image acquisition and FISH sequentially for Rho-GTP and Arp2 mRNA, respectively.

Although both fluorescent protein-based FRET and FISH are well established methods, the combination of them created several unique technical hurdles. The first challenge was how to preserve the RNA during the image acquisition for the FRET. In fact, at the beginning, we encountered almost total loss of Arp2 mRNA after the FRET images acquisition (data not shown). As described in "Materials and methods", we solved this problem by keeping the cell samples in the Fixation Buffer except when they were processed for FRET images. The second issue was that conventional cover slip mounting methods cannot be used because quick mounting and dismounting of the samples for FRET imaging is required to preserve the RNA. To solve this problem, we used a custom-made mounting device which allowed us to quickly mount and dismount the cover slip. The third issue is that the sequential process requires the identification of the same cells after FISH process for the Arp2 mRNA images. To meet this requirement, we used glass cover slips with engraved grids (Bellco) to help us to locate the same cells by their coordinates on the grids. In addition to the coordinates, we also used anti-

GFP antibodies to stain the YFP/CFP expressing cells after FISH to confirm the cells that were imaged for FRET.

Correlation of active RhoA and Arp2 mRNA localization

Having solved the above technical problems allowed us to detect RhoA activity and Arp2 mRNA in the same cells. We noticed that in normal growth, serum-starved and LPA stimulated cells, there is a relationship regarding the relative intracellular distribution between active RhoA and Arp2 mRNA. We categorized this relationship into three types: (1) uniform distribution where both active RhoA and Arp2 mRNA are globally distributed; (2) colocalized where both RhoA-GTP and Arp2 mRNA are relatively concentrated in the same region of the cell; and (3) excluded where RhoA-GTP and Arp2 mRNA are relatively concentrated in different regions of the cell (Fig. 6).

Because LPA induces RhoA activation (Fig. 3a) (Kumagai et al. 1993; Ridley 1994) and Arp2 mRNA localization (Fig. 1), we tested whether LPA stimulation would change the relative intracellular localization pattern of Rho-GTP and Arp2 mRNA. We transfected the cells with WT Raichu-RhoA and removed the serum to reduce the basal level of Arp2 mRNA localization, then stimulated the cells with LPA. In the absence of serum, about 40% of the cells showed localized Rho-GTP, among which about half exhibited co-localization of Rho-GTP and Arp2 mRNA while the other half presented a mutually exclusive localization (Fig. 7a). However, after LPA stimulation, the percentage of the cells showing mutual exclusion of Rho-GTP and Arp2 mRNA increased from ~20 to ~53% (Fig. 7a). To confirm this result of visual analysis, a more objective method was used to quantify overlap of the fluorescence intensity of the Arp2 mRNA with that of the localized Rho-GTP (see “Materials and methods”). This quantification demonstrates a statistically significant shift to less spatial overlap of Rho-GTP and Arp2 mRNA in each cell after LPA stimulation (Fig. 7b).

Discussion

In this report, we present data to demonstrate an LPA-induced, RhoA/ROCK mediated pathway for the localization of Arp2 mRNA in the fibroblasts. We have also described and demonstrated the application of a new method to detect FRET and mRNA in the same cells. This method has allowed us to reveal an interesting relationship of mutual exclusive localization of active RhoA and Arp2 mRNA in the fibroblasts in response to LPA stimulation. Historically, RhoA was believed to localize in the trailing edge of motile cells (Etienne-Manneville and Hall 2002; Worthylake et al. 2001). However, recent findings suggest that, RhoA is also activated at the leading edge in a number of cell types (Kurokawa and Matsuda 2005; Pertz et al. 2006; Stuart et al. 2008). Regardless of the causes of such discrepancy, these reports indicate that localized activation of RhoA plays an important role in cell functions such as polarity and directed cell migration (Goulimari et al. 2005).

Previous studies have demonstrated that RhoA and ROCK activities are required for localization of β -actin mRNA (Latham et al. 2001) and the enrichment of RNA contents in the pseudopods of tumor cells (Jia et al. 2005; Stuart et al. 2008). Our results that Arp2 mRNA localization requires RhoA and ROCK activities suggest a similar mechanism. However, a few observations suggest differences between the regulation of intracellular localization of β -actin mRNA and Arp2 mRNA. For example, β -actin mRNA localization is independent of microtubules (Sundell and Singer 1991; Latham et al. 2001) while Arp2 mRNA localization is dependent on microtubules (Mingle et al. 2005). Furthermore, constitutively active RhoA enhanced β -actin mRNA localization (Latham et al. 2001) but inhibited Arp2 mRNA localization (Mingle and Liu, unpublished). Given the different sensitivity to the activity level of RhoA, different dependence on the microtubules and that RhoA is a known regulator of

microtubules (Cook et al. 1998; Gundersen et al. 2005), a potential RhoA-microtubule pathway may be unique for the localization of Arp2 mRNA but not essential for β -actin mRNA.

By establishing a method to detect FRET and mRNA signals in the same cells, we were able to reveal LPA-induced mutual intracellular exclusion of RhoA-GTP and Arp2 mRNA. The mechanism behind such correlation is currently unknown. Given the observation that LPA induces Arp2 mRNA localization to the protrusions in fibroblasts, one would predict that RhoA activity is inhibited in the protrusions. Interestingly, Stuart et al. recently reported that RhoA activity in the protrusions is required for RNA enrichment in the protrusions of tumor cells (Stuart et al. 2008). It is not clear whether this discrepancy is due to the different cell types or experimental conditions. For example, it is unknown whether our observed mutually exclusive localization of RhoA-GTP and Arp2 mRNA represents a transient and rapid response to LPA stimulation. Nonetheless, our results reveal an interesting relationship of localized activation of RhoA and Arp2 mRNA localization, which provide a basis for future study of the dynamic spatial and temporal relationship between RhoA activity and Arp2 mRNA in live cell during migration. To our best knowledge, this is the first report in which FRET and mRNA are detected in the same cells. Our established experimental conditions for the sequential FRET and FISH should be easily adopted by other investigators for their choice of biosensors and DNA/RNA targets. There are limitations for our method which include that it works only with fixed samples and there is a time restraint for the FRET image acquisition because of the concern of RNA degradation. On the other hand, the fluorescence quenching of fluorescent protein biosensors by the FISH process may turn out to be an advantage because it opens the spectrum for the detection of multiple species of RNA in the same cells. Such potential for multiplex analysis will benefit the studies of complex questions in the broad field of cell biology.

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Fig. 1.

LPA stimulates Arp2 mRNA localization in fibroblasts. Cells were serum starved overnight and then stimulated with 2 µg/ml LPA for 10 min. **a** Representative images show Arp2 mRNA (red) and DAPI staining of the nucleus (blue) of cells with indicated treatments. Cells are outlined with *dashed lines* and *arrow* indicates localized Arp2 mRNA. **b** Quantification of at least 300 cells from three independent experiments. *Error bars* indicate SEM, *statistically significant $p < 0.01$ from no serum

Fig. 2.

Inactivation of Rho/ROCK pathway inhibits serum-mediated Arp2 mRNA localization. **a, c** Confirmation of C3 inhibiting the RhoA pathway by quantification of cells with intact stress fibers following transfection with the indicated constructs. Cells transfected with a 3:1 molar ratio of C3:GFP plasmid DNA were identified with GFP fluorescence and showed the expected percentage of cells with diminished stress fibers as a RhoA pathway indicator. At least 200 cells were counted from two independent experiments. **b, d** Quantification of Arp2 mRNA localization in CEFs that were transfected with GFP or co-transfected with C3 and GFP plasmid constructs at 3:1 ratio. In **b**, dotted lines indicate cell border and arrow points to localized cArp2 mRNA. * $p \leq 0.01$ from the cells transfected with only plasmid for GFP. **e** Quantification

of Arp2 mRNA localization in CEFs that were serum-starved overnight then treated with or without the indicated concentrations of Y-27632 for 30 min before serum stimulation with 10% FBS for two hours. For **d** and **e**, at least 300 cells from three independent experiments were analyzed for each condition. $*p \leq 0.01$ from 10% FBS control. *Error bars* indicate SEM

Fig. 3.

Inactivation of Rho/ROCK pathway inhibits LPA-induced Arp2 mRNA localization. **a** LPA induces activation of Rho in the CEFs. Rho-GTP pull down assay was performed as described in “Materials and methods”. Representative Western Blot samples for three independent experiments. **b** Inhibition of Arp2 mRNA localization by Rho inhibitor C3. CEFs were transfected with GFP or C3:GFP at 3:1 molar ratio then serum starved for ~18 h before stimulated with 2 µg/ml LPA for 10 min. Representative images for cells transfected with GFP or C3/GFP. **c** Inhibition of Arp2 mRNA localization by ROCK inhibitor Y-27632. CEFs were serum starved ~17 h then treated with 10 µM of Y-27632 for 30 min before stimulated with 2 µg/ml LPA for 10 min. In **b** and **c**, dotted lines indicate cell border and arrows point to localized cArp2 mRNA. **d** Quantitative results of LPA-induced cArp2 mRNA localization in cells transfected with GFP, C3/GFP, or treated with Y-27632. At least 300 cells from three independent experiments were analyzed for each condition. * $p \leq 0.001$ from positive controls. Error bars indicate SEM

Fig. 4.

Verification of the Raichu-RhoA constructs. **a** Representative images of YFP/CFP ratios of cells transfected with the indicated constructs. **b** Constitutively active and WT Raichu-RhoA constructs exhibits significantly higher FRET (YFP/CFP ratio) as compared to the dominant negative Raichu-RhoA construct. CEFs were transfected with corresponding Raichu-RhoA constructs and the YFP and CFP images were acquired and processed as described in “Materials and methods”. The obtained YFP/CFP ratio was presented as fluorescence intensity images and the total pixel intensity (arbitrary unit) per cell was quantified. For each construct: $n = 6$. Error bar SEM. $*p < 0.001$ and $**p < 0.01$

Fig. 5.

Quenching fluorescence of fluorescent proteins by FISH. CEFs were cultured on grid glass cover slips and transfected with indicated fluorescent protein expression plasmids. The cells were fixed 24 h after transfection and imaged. The samples then were further processed for in situ hybridization at 70°C over night followed by subsequent mimic of antibody incubation and tyramide signal amplification. Lower hybridization temperature (60°C) was also tested for eGFP and mCherry. After the FISH process, the same cells were identified and fluorescence images were acquired with identical parameters. Data are presented as the percentage of fluorescence intensity of the cells after FISH treatment compared to that of pre-FISH and

represent the quantitative results of 4–8 images for each condition and fluorescence protein from two independent experiments. *Error bar* SEM

Fig. 6. Three representative patterns of intracellular distribution of RhoA-GTP and Arp2 mRNA. Sequential FRET and FISH were processed for the detection of active RhoA and Arp2 mRNA, respectively (see “Materials and methods” for details). Anti-GFP antibodies were used to confirm the identity of the YFP/CFP expressing cells after the FISH process which quenches the fluorescence of the YFP and CFP. Arrows indicate regions of the cell where RhoA-GTP is relatively enriched

Fig. 7.

LPA stimulation enhances the percentage of cells showing mutually exclusive localization of RhoA-GTP and Arp2 mRNA. **a** Quantification of spatial distribution of RhoA-GTP and Arp2 mRNA by category illustrated in Fig. 6. **b** Quantification of spatial overlap of fluorescence intensity of RhoA-GTP and Arp2 mRNA. Thirty-seven cells were analyzed from four independent experiments. * $p = 0.017$ from using Kruskal–Wallis one-way analysis of variance