Lysophosphatidic acid signaling controls cortical actin assembly and cytoarchitecture in *Xenopus* embryos

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Summary

The mechanisms that control shape and rigidity of early embryos are not well understood, and yet are required for all embryonic processes to take place. In the *Xenopus* blastula, the cortical actin network in each blastomere is required for the maintenance of overall embryonic shape and rigidity. However, the mechanism whereby each cell assembles the appropriate pattern and number of actin filament bundles is not known. The existence of a similar network in each blastomere suggests two possibilities: cell-autonomous inheritance of instructions from the egg; or mutual intercellular signaling mediated by cell contact or diffusible signals. We show that intercellular signaling is required for the correct pattern of cortical actin assembly in *Xenopus* embryos, and that lysophosphatidic acid (LPA) and its receptors, corresponding to LPA₁ and LPA₂ in mammals, are both necessary and sufficient for this function.

Key words: Lysophosphatidic acid, Actin cytoskeleton, G-protein-coupled receptor, *Xenopus*

Introduction

The actin skeleton is required for many cellular processes, including cytokinesis, endocytosis and exocytosis; cell shape and polarity; cell process formation; and motility (Jacinto and Baum, 2003). During the egg-to-blastula stage in *Xenopus*, each cell assembles a cortical actin network of filament bundles, which is required for maintenance of overall rigidity and shape of the whole embryo (Kofron et al., 2002). In previous work we have shown that the cadherin-binding protein plakoglobin is necessary and sufficient for maintaining the cortical actin skeleton, and acts downstream of the cytoplasmic signaling intermediate cdc42 (Kofron et al., 2002). Loss of either of these proteins causes loss of shape and rigidity of the embryo, which collapses under its own weight. Examination of the cytoskeleton of such embryos reveals the loss of cortical actin, but not the microtubule or intermediate filament skeletons, of the blastomeres. Conversely, overexpression of cdc42 or plakoglobin increases the density of the cortical actin skeleton, and the rigidity of the embryo (Kofron et al., 2002).

Of particular interest is the mechanism by which each cell of the embryo assembles a similar cortical actin network. The number of cells increases rapidly by repeated cell divisions in the early embryo, and yet each cell, as it forms, assembles an actin skeleton appropriate to its contribution to the overall shape and rigidity of the whole embryo. In general, two mechanisms for this can be envisaged. First, each cell could inherit actin assembly instructions from the egg. Second, intercellular signaling could maintain the appropriate density and pattern of cortical actin filaments. In general, little is known about how cells of supracellular arrays all maintain actin skeletons appropriate for the shape, size and rigidity of the array. *Xenopus* embryos offer an attractive system in which to study this.

It has been known for many years that phospholipids can participate in intercellular signaling (Vogt, 1963), and their diverse roles have only recently been realized as more model systems have become available (Im et al., 2000; Yang et al., 2002). The phospholipid LPA can induce different cellular responses, depending upon cell type and context. These include smooth muscle contraction, cell proliferation, platelet aggregation, cell migration and neurite retraction (Goetzl, 2001; Xie et al., 2002). In particular, LPA signaling has been shown to influence both the actin cytoskeleton and cellular morphology. Increased LPA signaling in fibroblasts increases the formation of stress fibers. In different neural cell lines, it causes rapid process retraction, cell rounding or actin reorganization (Fukushima et al., 2002; Ridley and Hall, 1992; Yan et al., 2003). Overexpression of the *Xenopus* XLP₁ receptor in a rat neuroblastoma line that lacks endogenous LPA receptors, causes cell rounding, retracted neurites and an increase in stress fibers (Kimura et al., 2001).

LPA signals through G-protein-coupled receptors (GPCR) belonging to the rhodopsin-like class A receptors. These are seven transmembrane domain (TMD) proteins that bind specific G proteins to elicit responses (Anliker and Chun, 2004). The first LPA receptor was identified as a sheep orphan GPCR (Edg-2) and subsequently as the mouse ortholog of rec1.3 (Macrae et al., 1996; Masana et al., 1995). It was also identified in a screen for GPCRs associated with neuron production, as a transcript expressed in the ventricular zone of the developing mouse cortex, and demonstrated to be an LPA-specific receptor (Hecht et al., 1996). Overexpression of this
transcript in cell lines induced serum-dependent cell rounding, which was mimicked by addition of LPA. Verification that this was an LPA receptor was provided by studies in yeast and gain-of-function studies using the human ortholog (An et al., 1997; Erickson et al., 1998). Structural studies have suggested key residues to be important for phospholipid binding and LPA specificity (Wang et al., 2001). To date, three LPA receptors have been identified in mammals and renamed LPA₁, LPA₂ and LPA₃ (Lynch, 2002). These share sequence homology with a more divergent fourth receptor (Anliker and Chun, 2004). In *Xenopus*, a single LPA receptor and its pseudoallele have so far been identified. These are most closely related to mammalian LPA₁ (and designated here as XLPA₁A and XLPA₁B). Both genes are expressed maternally and throughout embryogenesis (Kimura et al., 2001).

In this work, we show that LPA signaling is both necessary and sufficient for maintenance of the normal cortical actin skeleton in the early *Xenopus* embryo. First, we show that an additional LPA receptor, most closely related to LPA₂ (designated here as XLPA₂) is expressed after the onset of zygotic transcription. No homolog of mammalian LPA₃ was identified. We show that either addition of LPA ligand, or overexpression of *Xenopus* LPA receptors, increases the density of the cortical actin network in the early embryo and increases the rate of wound healing. Conversely, depletion of XLPA₁ and XLPA₂ receptors in the blastula reduces the density of the cortical actin network. Cell disaggregation mimics the effect of LPA receptor depletion, and adding soluble LPA to dissociated cells reverses the effect. These data suggest an intercellular signaling mechanism for global patterning of the cortical actin network in the early *Xenopus* embryo.

Materials and methods

Oocytes and embryos

Ovaries were removed from mature females and stage VI oocytes were defolliculated and injected with antisense or morpholino oligonucleotides. For double injections, oocytes were incubated at 18°C for 24 hours after injection with antisense oligo and then injected with morpholino oligo. Oocytes were matured with 1 μM progesterone and fertilized using the host transfer technique as reported previously (Holw ill et al., 1987). Embryos were dejellied in 1% MMR solution (pH=7.8) and maintained in 0.1 M PBS+0.1% BSA. After 30 minutes with DNase I, purified by phenol:chloroform extraction and ethanol precipitation. Total RNA was isolated from either two oocytes or embryos at specified stages in a proteinase K solution as described (Kofron et al., 1997). Total RNA was incubated for 10 minutes in a lipid or control solution before analysis of cortical actin.

Oligonucleotides

Twelve antisense oligonucleotides complementary to both XLPA₁A and XLPA₁B mRNA were tested for their ability to deplete the maternal mRNAs by injecting into the marginal zones of oocytes, incubating for 24 hours at 18°C, and assaying for mRNA depletion using RTPCR. Antisense oligonucleotides that depleted both mRNAs to less than 20% of normal levels were phosphorothioate-modified, purified by HPLC, and resuspended in sterile, filtered water. The sequence of the oligo selected for use was as follows (where asterisks represent phosphorothioate linkages): LPA₁-10MP, 5’T*C*A*TT-GTAGTAGC*A*T*G*G*G 3’.

Morpholino oligonucleotides were designed that targeted both XLPA₁A and XLPA₁B or XLPA₂. These were resuspended in sterile, filtered water and injected at doses of 10-40 ng into either oocytes or embryos: XLPA₁A and 1B MO, 5’TTCACCTCAAGATGTACGTGCTG 3’; XLPA₂ MO, 5’ACCTACCATGTTACAGGCCAGCCT 3’.

RNA constructs

Clones encoding both *X. tropicalis* XLPA₁ and XLPA₂ were identified by blasting the murine sequences for LPA₃ against *X. tropicalis* cDNA libraries at the Sanger Institute site (http://www.sanger.ac.uk/). The following clones for XLPA₁ (TNeu092p02) and XLPA₂ (TNeu013j17) were isolated, sequenced and DNA was linearized with Asp718. Dominant-negative forms of the human small Rho GTPases were excised from the pKH3 vector (a generous gift from Yi Zheng) using BamHI and EcoRI and inserted into the pCS2+ vector. DNA was linearized with Apoll. In vitro transcription was performed using the SP6 mMessage Machine (Ambion). Samples were treated for 15 minutes with DNase I, purified by phenol:chloroform extraction and resuspended in sterile filtered water.

RT-PCR

Total RNA was isolated from either two oocytes or embryos at specified stages in a proteinase K solution as described (Kofron et al., 2002) and subsequently treated with DNase I. cDNA was synthesized using oligo dT primers from 1 μg total RNA. The cDNA samples were analyzed on the MJ Research Opticon. Uninjected samples were used to generate a standard curve for each primer set and all data were normalized to either ornithine decarboxylase or plakoglobin as a control. Water and no reverse transcriptase controls were run each time and found to produce no product. PCR reactions were run on a 1.8% agarose gel to verify amplification of the correct size fragment.

Analysis of the actin skeleton

Vitelline membranes were removed from stage 9 embryos in 1× MMR solution on agarose dishes. Embryos were fixed for 30 minutes with FG fix, washed with 1× PBS+0.1% Triton X-100 in PIPES buffer (Gard et al., 1997) before excision of animal caps to examine the undisturbed actin skeleton. Alternatively, animal caps were excised and cultured for 10 minutes before fixation in FG fix, to allow the analysis of the response of the actin skeleton to wounding. In each case, the cortical actin skeleton was analyzed exactly as described by Kofron et al. (Gard et al., 1997; Kofron et al., 2002). For lipid experiments, LPA, phosphatidic acid and phosphatidylethanolamine (Avanti Polar Lipids) were reconstituted in 0.4% lipid-free BSA (Sigma) in 1× MMR and 0.4% lipid-free BSA was added to all solutions as a carrier. After caps were cut, they were incubated for 10 minutes in a lipid or control solution before analysis of cortical actin.

Cell dissociation assays

Vitelline membranes were removed from mid-blastulae (stage 8). Five animal caps were cut, and dissociated in 67 mM phosphate buffer for 3 minutes (Snapec et al., 1987). Dissociated cells were transferred into 1× Ca²⁺/Mg²⁺-free MMR on a 1% agarose dish. After 1 hour, cells were transferred into 0.1-1 μM LPA in Ca²⁺/Mg²⁺-free MMR in glass dishes for five minutes, or allowed to reassociate in 1× MMR. Cells were resuspended in the LPA solutions and maintained in 1× Ca²⁺/Mg²⁺-free MMR for different time intervals before fixation. Cells were fixed for 4 minutes in FG fix, washed with 1× PBS+0.1%
Tween-20, and stained with Alexa 488-phalloidin. To determine if Ca\(^{2+}\) or Mg\(^{2+}\) affected the actin cytoskeleton of dissociated cells, the cells were transferred back into \(1 \times\) MMR 15 minutes after dissociation, incubated for 30 minutes, and fixed and stained as above.

Statistics
Using the Laser Scanning Microscope software (Zeiss), projections were made from z-stacks of single cells or animal caps. The mean intensity was recorded over a 5000 \(\mu m^2\) area for at least 15 dissociated cells in each group. For animal caps, the mean intensity was recorded over a 0.62 mm\(^2\) area for gain-of-function experiments and a 1000 cells in each group. For animal caps, the mean intensity was recorded over a 5000 \(\mu m^2\) area with the low threshold set to 100. The mean intensities were averaged and are reported as mean±s.e.m. Student’s t-test was used to determine significance and \(P<0.05\) was considered to be statistically significant.

Results
Actin-containing structures in cells of the *Xenopus* blastula
These were examined in fixed animal caps excised from *Xenopus* blastulae after fixation for 30 minutes in FG fixative. Alternatively, animal caps were excised and allowed to heal for 10 minutes before fixation. Figure 1 shows a dissecting microscope view of caps fixed before isolation (Fig. 1A) and after 10 minutes culture (Fig. 1B), by which time healing has started, the wound margins have become smooth and the outer surfaces of the caps are becoming visible as the cap rounds up. At the late blastula stage, each cell lining the roof of the blastocoel cavity had a dense cortical network of actin filament bundles (Fig. 1C,D) (see also Kofron et al., 2002). Cells extend occasional filopodia (arrowed in Fig. 1D). In caps that were allowed to heal for 10 minutes before fixation, actin-rich purse-strings formed around the margins of the caps (arrowed in Fig. 1E). In addition to forming a purse-string, cells in healing caps also extended many actin-rich processes, which obscured cell boundaries (Fig. 1E, see inset). Occasionally, cells were identified that had rounded up and were undergoing cytokinesis in the plane of the roof of the blastocoel (outlined in Fig. 1E). In these cells, actin rich contractile rings were seen (Fig. 1F). Outside the contractile rings, the cortical actin skeleton of a dividing cell was significantly less dense than that of controls, and was replaced by a coarser network of filament bundles (Fig. 1F).

Currently, the mechanism(s) by which each blastomere assembles these components, either in the intact embryo, or in response to wounding, is not known.

Intercellular signaling controls the density of the cortical actin network
Because each cell of the blastula has a similar pattern and density of cortical actin (Fig. 1C,D), we tested the possibility that intercellular signaling maintains or initiates this pattern. We removed animal caps from early blastulae and dissociated them into single cells by removing the divalent cations required for cell adhesion. The cells were kept apart, fixed after different times in culture and the cortical actin network stained using Alexa-488 phalloidin. The cortical actin network in dissociated cells changed over the course of 30-60 minutes from the dense cortical network seen in undissociated caps from sibling embryos (Fig. 2A,B), to a coarser network of thick filament bundles, similar to those of dividing cells in intact animal caps (compare Fig. 1F with Fig. 2C). To avoid the potential artifact that the actin skeleton is reduced by the Ca\(^{2+}/\)Mg\(^{2+}\)-free saline, we compared dissociated cells that had been cultured in Ca\(^{2+}/\)Mg\(^{2+}\)-free MMR before fixation with those that were transferred into \(1 \times\) MMR at low density after disaggregation for 30 minutes before fixation. There was no significant difference in the intensity of phalloidin staining in the two groups of cells (data not shown). Subsequent reaggregation of single cells by transfer at high density to \(1 \times\) MMR resulted in reassembly of the high-density cortical actin network characteristic of intact caps (Fig. 2D). This suggests that intercellular signaling, either through soluble ligands or by cell contact, is required to maintain the density and pattern of cortical actin assembly in each cell of the intact embryo.

LPA ligand and receptor are both functional in the *Xenopus* blastula
It is well established that LPA signaling influences the actin
cytoskeleton in many cell types in vitro. However, the functions of LPA signaling in vivo during embryogenesis are not well understood. To test whether it plays a role in the cortical actin network of early Xenopus embryos, we first carried out gain-of-function experiments using both the ligand and its receptors. Purified 18:1 oleyl-LPA, bound to lipid-stripped bovine serum albumin was added to animal caps isolated from late blastula and early gastrula stage embryos. Animal caps were excised, cultured for 10 minutes in the presence or absence of LPA, then fixed and stained for F-actin with Alexa 488-phalloidin (Fig. 3A).

In the presence of 1 µM LPA, there was a dramatic increase in F-actin in the cortical actin network throughout the animal cap and in the purse-strings (Fig. 3C). This resulted in faster healing in the LPA-treated animal caps (compare Fig. 3B with 3C). At high magnification, the actin network in LPA treated caps was thicker and less organized compared with controls, and cell boundaries were obscured by the abundance of actin in many regions (compare Fig. 3D with 3E). These effects were dose dependent in the range of 0.1-5 µM LPA. Two related phospholipids were used as controls for specificity. After treatment with 5 µM phosphatidic acid (PA), the caps either displayed no change or a slight decrease in cortical actin (Fig. 3F). PA-treated caps were flatter than controls, and there was no effect on the rate of wound healing. At higher magnifications, the cortical actin network was similar in density to control embryos and the cells contained similar patterns of F-actin (not shown). Phosphatidylethanolamine (PE) had no effects, either on wound healing or on the cortical actin network (not shown). There was a significant increase in the intensity of phalloidin staining from 878±112 to 1154±160 in the 1 µM LPA treatment group, but no change with 5 µM PA (735±62) (Fig. 4A). All data represents four independent experiments with five caps per group in each experiment. These data show that LPA is sufficient to increase cortical actin at early blastula stages, and this suggests the receptors for LPA signaling are present and functional in the embryo.

Identification of a second LPA receptor in early Xenopus embryos

Two genes encoding LPA receptors have been described in Xenopus laevis: XLPA1A and XLPA1B (Kimura et al., 2001). These both show 90% homology to the human LPA1 receptor and are 98% identical in amino acid sequence to each other. As Xenopus laevis is allotetraploid, these are most likely pseudoalleles and represent the duplicated orthologs of the
mammalian LPA$_1$ receptor. The sequences for murine LPA$_2$ and LPA$_3$ were used to screen the X. tropicalis cDNA databases at the Sanger Institute (http://www.sanger.ac.uk/). Two X. tropicalis clones were identified (TNeu013j17 and TGas026e21) with significant homology to mouse LPA$_2$. These were obtained and sequenced and found to encode the same mRNA. As only TNeu013j17 contained the full coding sequence, this was used for experiments described here. The full sequence of X. tropicalis XLPA$_2$ mRNA was deposited into GenBank as Accession Number AY652941.

The predicted protein was found to be 62% identical and 16% similar to mouse LPA$_2$ at the protein level and thus was designated X. tropicalis XLPA$_2$. It contains 344 amino acids, has a predicted molecular mass of 39.5 kDa, and is predicted to have seven putative transmembrane domains (TMD) (Fig. 5A). XLPA$_2$ is most divergent from the mammalian orthologs in the fourth and fifth TMDs and at the C terminus. Based on structural models, LPA receptors have been shown to contain three residues that interface with LPA (Wang et al., 2001). XLPA$_2$ contains the conserved arginine and lysine in the third and seventh TMD, respectively, that are thought to interact with the head group of LPA; and a glutamine in the third domain that confers LPA specificity (highlighted in red in Fig. 5A). Like mammalian LPA$_2$ receptors, it also lacks the longer extracellular N terminus of LPA$_1$.

No orthologs of mouse LPA$_3$ were found in egg, gastrula, neurula or tadpole libraries.

**Expression of LPA receptors during Xenopus laevis development**

Total RNA was isolated from a series of developmental stages, and expression levels of XLPA$_1$ and XLPA$_2$ analyzed by real-time RTPCR. As reported previously, XLPA$_1$ was found to be most abundant in the oocyte (Kimura et al., 2001). After the mid-blastula transition (MBT) and the onset of zygotic transcription, levels of the XLPA$_1$ transcript fall, but low levels of XLPA$_1$ expression continued until at least stage 45. Conversely, XLPA$_2$ mRNA was not detected in oocytes or early embryos. Expression commenced at MBT, and remained constant until at least stage 45 (Fig. 5B). Results are representative of a single experiment and all experiments were repeated at least three times with five caps per group.

**Overexpression of X. tropicalis LPA receptors mimics addition of LPA ligand**

To assay for the presence of functional LPA ligand at the blastula stage, we injected 400 pg of either XLPA$_1$ or XLPA$_2$ receptor mRNA at the two-cell stage (200 pg/blastomere), and excised animal caps for analysis of the actin skeleton at the late blastula stage. After removal of the vitelline membrane, embryos injected with either XLPA$_1$ or XLPA$_2$ became elongated along the animal-vegetal axis (Fig. 6A,B). They were also more compact than controls, and the animal caps healed faster than control caps (Fig. 6C). The effects on animal caps of LPA receptor overexpression were identical to those caused by addition of LPA to the animal caps; denser networks of cortical actin, thicker purse-strings, no change in contractile rings and faster wound-healing (Fig. 6D,E). Overexpression of LPA receptors caused a significant increase in phalloidin intensity over a 0.62 mm$^2$ area from 1133±177 to 1372±302 or 1610±348 for XLPA$_1$ and XLPA$_2$, respectively (Fig. 4B). All data represent four independent experiments with five caps per group. Therefore, overexpression of the LPA receptor is sufficient to increase cortical actin and the rate of wound healing in the early embryo, and demonstrates the presence of endogenous ligand.

**LPA signaling is necessary, as well as sufficient, for cortical actin polymerization in the Xenopus blastula**

The presence of a maternal store of LPA$_1$ mRNA in the oocyte suggested that LPA signaling may be controlled, at least until the blastula stages, by maternally encoded genes. So, for loss of function experiments, we first depleted the stored maternal LPA$_1$ mRNA using antisense oligodeoxynucleotides. Twelve oligos, each complementary to both XLPA$_{1A}$ and XLPA$_{1B}$, were synthesized and tested for their ability to deplete both
XLPA1 mRNAs after injection into the oocyte cytoplasm. One was selected and modified by replacing the 5′ and 3′ phosphodiester linkages with phosphorothioate linkages. Doses of 5, 7.5 and 10 ng were injected into manually defolliculated full-grown oocytes, which were fertilized 48 hours later by the host transfer technique (Holwill, 1987). XLPA1 mRNA levels were reduced to 16% of control levels at the two-cell stage (Fig. 7A). At stage 10, XLPA1 mRNA in the controls had decreased significantly, resulting in a relative increase in the depleted embryos to 33% of control levels.

The cortical actin in late blastula embryos was assayed either by fixation before removal of the animal cap, or by fixation 10 minutes after excision of the animal cap (to assay the response to wounding). In both cases, levels of cortical actin, including the purse-string that formed in response to wounding, as well as cortical actin in each cell, were reduced, compared with control embryos. Animal caps from depleted embryos, and the bases from which they were excised, healed more slowly than controls (Fig. 7B,C). At high power, cells in LPA1-depleted animal caps were found to have lost the dense cortical network of actin filament bundles, but retained a coarser network similar to that seen in dividing cells, and in dissociated cells. In addition, fewer cell processes were present (Fig. 9B). These data are representative of four independent experiments with five animal caps per group.

Depletion of XLPA2 by the morpholino oligo had no effect before the onset of zygotic transcription, consistent with the fact that onset of transcription starts at the mid-blastula stage (Fig. 5). However, at late blastula and early gastrula stages, it caused effects similar to depletion of XLPA1 (Fig. 9B). Injection of 15 ng of both morpholino oligos together caused effects similar to 40 ng of either morpholino alone (Fig. 9B). Injection of both morpholino oligos together caused effects similar to 40 ng of either morpholino alone (Fig. 9B). Data are representative of three independent experiments with five animal caps per group. These experiments show that signaling through XLPA1 is required to maintain the normal density of the cortical actin network in the early Xenopus embryo.

Despite the reduction of cortical actin at the blastula stage, embryos depleted only of the maternal XLPA1 were able to gastrulate and develop normally to tadpole stages (Fig. 7E). This could be due to re-establishment of receptor levels as the maternal store is replaced by zygotic transcription of XLPA1 and/or XLPA2. To test this possibility, we synthesized antisense morpholino oligos, which block translation of their target mRNAs throughout early development (Heasman et al., 2000), complementary to each mRNA separately (XLPA1-MO and XLPA2-MO). These were injected at either the two-cell stage of development into the animal hemisphere at doses from 10-40 ng, or into oocytes that were then fertilized using the host transfer technique.

At doses of 20-40 ng of the XLPA1-MO, there was a generalized decrease in the amount of F-actin staining throughout all cells in the animal caps (Fig. 9A), similar to caps depleted of maternal XLPA1. Purse-strings were present after animal cap excision, but at reduced levels compared with control caps (Fig. 9A). At high power, cells in LPA1-depleted caps were found to have lost the dense cortical network of actin filament bundles, but retained a coarser network similar to that seen in dividing cells, and in dissociated cells. In addition, fewer cell processes were present (Fig. 9B). These data are representative of four independent experiments with five animal caps per group.

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embryos that were injected with XLPA1 or XLPA2 morpholino oligos, which block translation of the zygotic mRNA as well, did show later developmental defects. These were first evident during gastrulation (Fig. 9C), which proceeded more slowly, with blastopores remaining open longer than those of control embryos. Defects became more severe during neurulation (Fig. 9D), with defects ranging from slower closure of the neural folds, to significantly reduced neural fold formation. By the tail-bud stage (Fig. 9E), LPA1 or LPA2-depleted embryos showed reduction in body length and major defects in many organ rudiments. These pleiotropic effects are most likely due to an expanding number of LPA-mediated morphogenetic events during later stages.

**Addition of soluble LPA to isolated cells restores the cortical actin density to in vivo levels**

Loss of LPA signaling reduces the density of the cortical skeleton, and mimics the effect of dissociating the cells, suggesting that LPA is an endogenous intercellular signal that controls the density of the cortical actin skeleton. To test this, cortical actin skeletons were compared between intact embryos, cells from embryos that had been dissociated at the mid-blastula stage and kept apart for 1 hour, and cells kept apart for 1 hour and then incubated for 5 minutes in 0.1 or 1 µM LPA. The cortical actin skeleton was significantly reduced in dissociated cells compared with intact embryos, and was rescued by subsequent addition of LPA to the dissociated cells (Fig. 10A). The mean fluorescence intensity for each cell was determined over a 5000 µm² area and averaged for each group. Addition of LPA to dissociated cells caused a statistically significant rise from 933±180 to 1626±349. Washing out the LPA, and keeping the cells dissociated caused a drop in cortical actin back to the level in dissociated cells after 45 minutes (Fig. 10B). The experiment was repeated three times with the same result. These data show that continuous signaling by LPA is required to maintain the normal pattern and level of cortical actin.

**Dominant negative Rho and Rac, but not cdc42, block the overexpression effects of LPA receptors**

The effects of LPA on the actin cytoskeleton in Swiss 3T3 fibroblasts are mediated through the small Rho GTPases, including activation of RhoA and Rac1. A dominant-negative form of RhoA (RhoA-N19) blocked the formation of stress fibers in response to LPA, while the formation of lamellipodia was blocked by expression of a dominant-negative Rac1 (Rac-N17) (Ridley and Hall, 1992; Ridley et al., 1992).

To determine whether LPA signaling in early Xenopus embryos acts through similar pathways, we expressed these same dominant-negative constructs, assayed their effects on the actin skeleton and asked if overexpression of LPA receptors could rescue these effects. We injected mRNA for either XLPA2 alone, a dominant-negative GTPase alone or for both mRNAs at the two-cell stage and analyzed the actin skeleton at stage 9. Overexpression of RhoA-N19 alone resulted in a loss of purse-strings (arrow in Fig. 8B, upper middle panel), delayed wound healing and an increase in cellular processes (Fig. 8A,B, lower left panel). At higher doses, cell division was blocked and occasionally large cells were seen that had not divided (not shown). When XLPA2 and RhoA-N19 were injected together, the Rho-N19 blocked the effect of XLPA2 on wound healing, but not the increase in overall cortical actin (Fig. 8A,B). This suggests that RhoA is downstream of LPA signaling in the formation of purge strings and wound healing, but not in the pathway leading to assembly of the cortical network of actin.

Overexpression of Rac-N17 alone also resulted in loss of purse-strings (Fig. 8A, upper right panel). In addition, there was a dramatic loss in the cortical actin network in each cell (Fig. 8B, lower middle panel). Co-injection of XLPA2 mRNA did not rescue this effect, showing that Rac is downstream of
LPA signaling leading to assembly of the cortical actin network (Fig. 8A,B). When XLPA2 was co-injected with dominant-negative forms of cdc42, there was no blockade of the overexpression effects of XLPA2 (data not shown).

Discussion

The data presented show that intercellular signaling is required to maintain the normal cortical actin pattern and density in each blastomere during early Xenopus development, and that LPA signaling is both necessary and sufficient for this. LPA is a bioactive lipid, known to be involved in intercellular signaling. It is generated outside the cell by ectoenzymes, and acts upon specific G-protein-coupled receptors. Four LPA receptors have been identified in humans and mice. These have been known previously by a variety of names and re-classified more systematically recently as LPA1-4 (Chun et al., 2002). In Xenopus, one receptor, with high homology to LPA1, has already been identified (Kimura et al., 2001). We report here a second receptor with high homology to mammalian LPA2. Interestingly, XLPA1 is stored as a maternal mRNA, while XLPA2 commences expression at the mid-blastula stage. As the experiments described here suggest they play redundant roles in maintaining the actin skeleton, it is interesting that they are not coordinately regulated at these early stages.

LPA-mediated signaling has been implicated in a wide range of cell behavior, including proliferation, survival, motility, cell shape and differentiation (Anliker and Chun, 2004; Fukushima et al., 2002; Tigyi, 2001; Ye et al., 2002). Targeted mutation of LPA receptors in the mouse has shown that LPA signaling is required for normal development (Contos et al., 2000; Contos et al., 2002). Redundancies in receptor function and the pleiotropic effects of their removal have made it difficult to identify specific cellular events in specific organs that require LPA signaling. However, it is clear that in its absence, normal development does not occur. One specific event found to require LPA signaling in vivo was survival of Schwann cells in the sciatic nerve (Contos et al., 2000). In the present study, we have used the early Xenopus embryo as a relatively simple and tractable system to identify a specific role for LPA signaling in vivo. Upregulation of either the ligand or its receptor increased the density of cortical actin, indicating the presence of functional receptor and ligand in the embryo. Downregulation of the two LPA receptors had the converse effect, indicating that LPA signaling is both necessary and sufficient for maintenance of the correct density and pattern of cortical actin.

It is of interest that either dissociation of the blastula cells or depletion of the LPA receptors, caused loss of the high-density cortical actin network, but left a coarser network of actin filaments remaining in the blastomeres. When LPA is added to dissociated cells, or they are allowed to aggregate again, a denser network, similar to that found in vivo, was assembled. This suggests that there are cell-autonomous
mechanisms, either mediated by autocrine signaling or constitutively active signaling intermediates, that maintain a basal level of actin assembly, and LPA signaling between cells converts this to the dense network seen in cells that are connected to other cells in the embryo. In this context, it is interesting that cells rounding up to divide lose the denser network, suggesting that LPA signaling may be switched off to allow them to do this. At the moment, we have no direct evidence for this hypothesis, nor of its mechanism.

Intercellular signaling can be mediated through cell-cell contacts, secreted signals that function in an autocrine or paracrine fashion, or both. It has been shown that cell-cell contacts, in particular adherens junctions, modulate the cortical actin skeleton (Gumbiner, 1990; Gumbiner, 1996). In this work, we have not determined the roles of adherens junctions. However, the loss-of-function data presented here shows that LPA signaling is a necessary signal for regulating the density of the network. In dissociated cells, LPA is sufficient to increase the density of the actin cytoskeleton without cell contact. In addition, loss of LPA receptors in the whole embryo results in a coarser network, without affecting cell adhesion, suggesting that cell-cell contacts are still present. Despite this, it is likely that cell junctions will provide information to the cell, in addition to intercellular lipid signaling, to establish the correct pattern and density of actin filaments.

We find that there is redundancy in signaling through the XLPA\textsubscript{1} and XLPA\textsubscript{2} receptors with respect to the changes in the actin cytoskeleton. Both receptors, when overexpressed, produced a similar increase in cortical actin and more rapid wound healing. In addition, a high dose of each morpholino individually caused a similar phenotype to a lower dose of both morpholinos together. This suggests that the quantity, rather than the nature, of LPA receptors is crucial for the actin cytoskeleton, and that one receptor may compensate for the other. No late developmental phenotype was apparent when the phosphorothioate oligo was used to deplete only the maternal store of XLPA\textsubscript{1}. This was most likely due to the onset of XLPA\textsubscript{1} and XLPA\textsubscript{2} production after the MBT. Redundancy also exists between murine LPA receptors. The Edg4\textsuperscript{-/-} mouse (mouse homologs of LPA\textsubscript{1} and LPA\textsubscript{2} are known as Edg2 and Edg4, respectively) showed no obvious gross or histological phenotype and the Edg2\textsuperscript{2+/+}/Edg4\textsuperscript{-/-} mouse only showed an increase in frontal hematomas compared with the Edg2\textsuperscript{2+/+}/Edg4\textsuperscript{-/-} mouse (Contos et al., 2002). In addition, when LPA was added to mouse embryonic fibroblasts isolated from the meninges, stress fibers formed throughout the cell. This response was only blocked in fibroblasts isolated from the Edg2\textsuperscript{2+/+}/Edg4\textsuperscript{-/-} mouse and not from the individual knockouts (Contos et al., 2002).

It is likely that LPA signaling is required for more than the formation of the cortical actin skeleton in the blastula. It is an advantage of this model system that the function in cortical actin skeleton can be studied at an early stage, in the absence of a background of pleiotropic roles of LPA. However, the extensive later developmental defects caused by blockade of LPA\textsubscript{1} and LPA\textsubscript{2} suggest that LPA signaling is required in different regions of the embryo as more cell types form, and multiple types of cell behavior develop. It will be of interest to identify these, and
the mechanisms whereby LPA signaling is spatially and temporally controlled during embryogenesis.

LPA receptors require the function of the small Rho GTPases XRho and XRac to elicit the overexpression effects of increased cortical actin, increased wound healing and thick animal caps. It has been well established that in many cell types LPA signaling functions through RhoA in a \( \gamma_{12/13} \) pathway (Contos et al., 2002; Kimura et al., 2001; Ridley and Hall, 1992; Yan et al., 2003). Additional evidence demonstrates that LPA may also activate Rac through a \( \gamma_{i/o} \)-mediated pathway to exert its effects (Van Leeuwen et al., 2003). Although we have not determined which G proteins are used in our model, it is possible that XRho and XRac are being activated in the embryo by similar mechanisms as in single cells.

Both addition of LPA to animal caps and overexpression of either LPA receptor increased the rate of wound healing. One mechanism that LPA may be affecting is assembly of a purse-string. Brock et al. first described the formation of an actinomyosin purse-string that is assembled rapidly to provide the driving force to close embryonic wounds (Brock et al., 1996). However, previous work in Xenopus embryos suggests that in superficial wounds, where the deep layer of cells is not breached, the purse-string does not provide the driving force for wound closure (Davidson et al., 2002). Instead, contraction and ingression of the deep cells may pull the wound closed. The results presented here do not discriminate between purse-string-mediated and non-purse-string-mediated mechanisms of wound healing. They show only that LPA signaling is required for normal purse string assembly and for wound healing.

It has been hypothesized previously that LPA signaling may play a role in wound healing. Regular application of LPA to a surface wound in a rat model accelerated wound closure and a thickening of the epithelial layer after wounding (Balazs et al., 2001). In our gain-of-function experiments, the thickness of the animal cap was increased in a similar manner and the caps rounded up faster than controls. In loss-of-function experiments...
experiments, wound healing was delayed, but the embryo could still heal. It is possible that there are redundant signaling systems that compensate for the loss of LPA signaling during wound healing, such as signaling by related phospholipids. In the Edg2+/Edg4+- mouse, normal wound healing was observed compared with control mice, but this may also due to functional redundancy and complexity of the mouse model (Contos et al., 2002).

In Drosophila, substantial changes in cell shape by the leading edge cells are required to draw the wound closed, while in final stages filopodia between cells may bridge the wound and assist in closure (Wood et al., 2002). In Xenopus oocytes, wound closure is mediated by drawing the wound closed in a circular fashion via an actinomysin purse string composed of F-actin and myosin II (Bement et al., 1999). The signals that control these responses have yet to be elucidated. The experiments documented here show that LPA signaling is required in vivo for cellular responses to wounding in the early Xenopus embryo.

In conclusion, these experiments show that intercellular signaling by LPA and its two receptors provides an essential mechanism for coordinating the pattern and density of actin assembly in individual cells of a supracellular array as it forms from a single cell, thus controlling its overall architecture and rigidity. This mechanism is likely to be used many times in development to generate specific architectural shapes from groups of individual cells.

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References
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