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Abstract

The phospholipid, lysophosphatidic acid, signals through G-protein coupled receptors to influence cell morphology, gene expression, cell survival, and cell proliferation in many cell types in vitro. Determining the role of LPA signaling in mammalian models has been difficult due to a significant amount of redundancy in this subclass of receptors. We use Xenopus laevis as a model because there is less redundancy and reagents may be targeted to specific tissues. The first goal of this study was to determine the role of LPA signaling in modulating actin dynamics in Xenopus. We show here that intercellular signaling is required to maintain an actin skeleton of appropriate density. LPA signaling is both necessary and sufficient to control the density of the cortical actin cytoskeleton during the blastula stage. These effects may be attributed to signaling through either of the Xenopus receptors, XLPA₁ or XLPA₂. We propose that LPA is an intercellular signal that may control the change from a sparse to dense actin network. The second goal of this study was to demonstrate specific roles for XLPA₁ at later stages. During neurulation, signaling through XLPA₁ is necessary as a survival and proliferation signal in the anterior CNS. These effects are not due to changes in the actin cytoskeleton and not attributable to XLPA₂. These results demonstrate that LPA signaling has necessary roles during early development and that there are both distinct and redundant roles for individual LPA receptors.
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ABBREVIATIONS

C1P  Ceramide-1-phosphate
CNS  Central nervous system
DAG  Diacylglycerol
DRG  Dorsal root ganglion
Edg  Endothelial differentiation gene
ER   Endoplasmic reticulum
ERK  Extracellular signal-regulated kinase
ExoC3 Exoenzyme C3
GAP  GTPase activating protein
GPCR G protein-coupled receptor
IP3  Inositol triphosphate
JNK  c-Jun N-terminal kinase
KO   knockout
LPA  Lysophosphatidic acid
LPP  Lipid phosphate phosphohydrolase
MAPK Mitogen-activated protein kinase
MEF  Mouse embryonic fibroblast
Mil  Miles apart
PA   Phosphatidic acid
PI3K Phosphatidylinositol-3'-OH kinase
PLC  Phospholipase C
PTX  Pertussis toxin
RLDX Rhodamine lysine dextran
ROCK Rho-associated kinase
S1P  Sphingosine-1-phosphate
Vzg1 Ventricular zone gene 1
CHAPTER I

INTRODUCTION
Lysophosphatidic acid (LPA)

The phospholipid, lysophosphatidic acid (LPA), is an abundant component of serum. It has been known for many years that phospholipids play critical structural roles in cell membranes, but only recently have signaling roles been attributed to them. LPA was first shown to have such roles in the late 1970s, causing three robust responses: platelet aggregation, uterine smooth muscle contraction, and alterations in blood pressure (Gerrard et al., 1979; Schumacher et al., 1979; Tokumura et al., 1978; Tokumura et al., 1980). It was quickly appreciated that LPA can play different roles based on the location in which it is produced. As an intracellular phospholipid, LPA serves as a key intermediate in the biosynthesis of membrane glycerophospholipids, whereas extracellularly, it influences cell morphology, architecture, migration, survival, proliferation, and differentiation. Additionally, an intracellular signaling role has been proposed for LPA, in which it may signal through complexes in intracellular compartments, but this is unlikely and has not been tested (Anliker and Chun, 2004; Moolenaar et al., 2004).

LPA describes not one, but a group of phospholipids. In human plasma, an array of different forms of LPA, including linoleoyl, arachidonyl, and oleoyl, are most abundant, but small amounts of both the palmitate and oleate forms are detected in some fluids (Baker et al., 2001). LPA is based upon a glycerol backbone with the phosphate monoester at position 3 (Lynch et al., 1997; Lynch and Macdonald, 2002). A single long chain fatty acid of at least 12 carbons may be attached to either the 1st or 2nd carbon position, but usually occupies position
To add further diversity, LPA may differ by the linkages at the first or second carbon (acyl, alkyl, and alkenyl bonds) and in the degree of saturation of the long chain fatty acid (Xie et al., 2002a). In physiological contexts, linkage to carbon 1 is more common, but in pathological states, such as ovarian cancer, LPA species with the fatty acid on position 2 are found in peritoneal ascites fluid (Xu et al., 1995).

Sphingosine-1-phosphate (S1P) represents a class of sphingosine-based phospholipids that are structurally similar to LPA, having a phosphate at the 3rd position and an alkenyl linkage on position 1. S1P differs from LPA primarily by the presence of an amine at the 2nd position (Fig. 1B) (Spiegel and Milstien, 2003).

In serum, LPA concentration ranges from 200 pM to 20 µM (Jalink et al., 1995; van Corven et al., 1989). LPA within the endoplasmic reticulum (ER) and mitochondria is converted rapidly to phosphatidic acid (PA), a precursor to membrane phospholipids such as phosphatidylcholine and phosphatidyl-ethanolamine. LPA synthesized outside the cell by ectoenzymes cannot cross membranes due to its amphipathic nature, so its function is limited to the cell surface (Pages et al., 2001). Extracellular LPA is detected in human and rat plasma, ocular fluid, rat cerebrospinal fluid, and in conditioned medium from prostate cancer cell lines (Xie et al., 2002b). In the extracellular compartment, LPA is either bound to serum albumin ($K_d$ 360 nM) or to plasma gelsolin released due to cellular injury (Goetzl et al., 2000; Tigyi et al., 1991).
Figure 1. Structures of LPA and S1P.

Figure 2. Homology between GPCRs. A. Phylogenetic analysis of mouse LPA, S1P, melanocortin 3, and cannabinoid 1 receptors. LPA1 and S1P1 cluster closest, then melanocortin3, and finally cannabinoid1. B. Alignment of LPA1 and S1P1. Conserved residues required for LPA binding are shown in blue. The residue in red confers specificity for binding either LPA or S1P.
A class of G protein-coupled receptors mediates LPA signaling

Extracellular LPA signals through G protein-coupled receptors (GPCR), which are seven transmembrane domain-containing proteins with extracellular N-termini and intracellular C-termini. The LPA receptors cluster under class A (rhodopsin), which includes catecholamine, opioid, small peptide, and many orphan receptors. The S1P receptors are most closely related to the LPA receptors, and more distantly to melanocortin and cannabinoid receptors (30% and 32% homology on the amino acid level respectively, Fig. 2A). In mammals, three closely related LPA receptors have been identified along with a more distantly related fourth receptor (LPA$_{1.4}$). LPA$_1$ and LPA$_2$ do not show selectivity for ligand based on the position or saturation of the fatty acid, but LPA$_3$ prefers 2-acyl species and unsaturated fatty acids (Sardar et al., 2002).

Binding studies carried out by several groups demonstrate that three amino acid residues are critical for LPA binding and association with its receptors. These include basic amino acids in the third and seventh transmembrane domains respectively (Fig. 2B, blue arrows), necessary for LPA binding, and a glutamine that is essential to confer LPA specificity. Substitution of the glutamine with a glutamate residue (Fig. 2B, red arrow) will change the affinity of LPA receptors to S1P from LPA by stabilizing the S1P amine group (Sardar et al., 2002; Wang et al., 2001). While the receptors have high homology among the transmembrane domains, LPA$_1$ contains a longer extracellular N-terminus than LPA$_2$ and diverges most in the C terminus, where the proteins are likely to interact with intracellular signaling complexes. The C-terminus is
proposed to function in receptor internalization and desensitization, processes common to this class of GPCR. The C terminus also contains a PDZ domain that may function to assemble signaling complexes at the membrane (Anliker and Chun, 2004).

The first LPA receptor, identified in 1995, was a sheep orphan GPCR named endothelial differentiation gene 2 (edg2). A mouse ortholog (rec1.3) was identified in 1996 (Macrae et al., 1996; Masana et al., 1995). Later, a screen was performed for GPCRs that are associated with neuron production, in which a transcript, named ventricular zone gene 1 (vzg-1), was found to be elevated in the ventricular zone of the developing mouse cortex and identified as a bona fide LPA receptor (Hecht et al., 1996). After a logical nomenclature system was devised, this receptor was renamed LPA₁ for the ligand it binds (Chun et al., 2002). Two more class members were identified subsequently based on structural homology, signature LPA residues, and responsiveness to LPA. LPA₁ and LPA₂ are the most closely related receptors in the class, and it is thought that these may have arisen from a common ancestor (Fukushima and Chun, 2001). Recently, a fourth LPA receptor was identified, P2Y9/ GPCR23, which utilizes LPA as a ligand, but this gene is phylogenetically separate from the other GPCRs, and GPCR23 may function through different downstream signaling mechanisms. GPCR23 has 20-24% homology to the other LPA receptors on the amino acid level, and clusters closer to the purinergic receptors (Noguchi et al., 2003).
Intracellular pathways activated by LPA signaling

Downstream of LPA receptors, several classes of G proteins are activated, leading to a variety of responses. The LPA\textsubscript{1} and LPA\textsubscript{2} receptors activate the G\textsubscript{i/o}, G\textsubscript{q}, and G\textsubscript{12/13} families (Figure 3). LPA\textsubscript{3} activates only G\textsubscript{i/o} and G\textsubscript{q} while GPCR23 couples to G\textsubscript{s}. Activation of G\textsubscript{i/o} leads to recruitment and activation of phosphatidylinositol-3'-OH kinase (PI3K) and Akt (PKB) resulting in cell survival, stimulation of the small Rho GTPase Rac resulting in proliferation and actin changes, activation of mitogen-activated protein kinase (MAPK) pathways, and inhibition of adenylate cyclase. The global effects of G\textsubscript{i/o} activation are cell survival, proliferation, and cytoskeletal changes. Stimulation of G\textsubscript{q} causes phospholipase C (PLC) activation, production of the second messengers diacylglycerol (DAG) and inositol triphosphate (IP\textsubscript{3}), and intracellular calcium mobilization. In some cell types, this response may influence gene expression. Finally, activation of G\textsubscript{12/13} activates the small Rho GTPase Rho, and subsequently Rho associated kinase (ROCK), to promote actin-myosin interactions and to form stress fibers (Anliker and Chun, 2004). Crosstalk between these intracellular pathways may occur, as both Rho and Rac are activated by separate pathways, but these pathways converge to regulate actin dynamics (Fig. 3). Additionally, it has been reported that Rac has a negative influence on Rho activation by increasing reactive oxygen species that will in turn...
Figure 3. Intracellular pathways activated by LPA and S1P receptors. Activation of G12/13 pathways lead to increased Rho and ROCK activity. Activation of Gi/o leads to PI3K, Akt, and MAPK family member stimulation and inhibition of adenylate cyclase. Activation of Gq causes increased calcium signaling.
activate 190RhoGAP (GTPase activating protein) to reduce the amount of active Rho-GTP (Moolenaar et al., 2004).

To clarify which pathways are downstream of specific receptors, in vitro studies have been carried out using mouse embryonic fibroblasts (MEFs) isolated from knockout animals. MEFs express both LPA₁ and LPA₂, but not LPA₃. They also express S1P₁, S1P₂, and S1P₃. Addition of LPA to MEFs causes calcium increases, as detected by measuring increases in IP₃ and rises in intracellular calcium using Fura-2/AM as a tracer. It also causes inhibition of adenylate cyclase, based on the inability of forskolin to induce cAMP accumulation. MEFs treated with LPA increase rates of cell proliferation, determined by BrdU incorporation, and both Akt and c-jun N-terminal kinase (JNK) are activated, demonstrated in western blots detecting the phosphorylated forms of these proteins. On the other hand, the MAPK family member extracellular signal-regulated kinase (ERK), is not diphosphorylated in response to an LPA signal in MEFs. Finally, Rho activation results in stress fiber formation after the addition of exogenous LPA (Contos et al., 2002).

In MEFs isolated from the LPA₁ knockout (KO) mouse, there is a block in activation of the PLC pathway, an inhibition of calcium increases, and a complete loss of adenylate cyclase inhibition. In MEFs from the LPA₂ KO mouse, there is also a considerable decrease in PLC activation, but no change in adenylate cyclase activity. Therefore, inhibition of adenylate cyclase is attributed to the LPA₁ receptor. In MEFs isolated from mice with targeted deletion of both receptors, there is decreased activation of JNK and Akt and loss of Rho...
activation in addition to the above phenotypes, suggesting that the receptors play
redundant roles in these functions. Therefore, there are several individual and
overlapping roles for LPA₁ and LPA₂, including the possibility that they both
activate these pathways synergistically (Contos et al., 2002).

**LPA may influence cytoskeletal dynamics**

Responses to LPA have been studied primarily in cell lines, and thus
many potential roles for these receptors have been determined *in vitro*. In an
immortalized mouse neuroblast line that lacks LPA receptors, overexpression of
LPA₁ and subsequent LPA treatment induces soma rounding and rapid neurite
retraction. In similar gain-of-function assays, using rat neuroblastoma lines that
lack endogenous LPA receptors (Hecht et al., 1996), overexpression of LPA₁ and
LPA treatment causes neurite retraction and the formation of stress fibers
throughout the soma (Fukushima et al., 1998). When cells are incubated in
clusters, additional behaviors are present. LPA induces the cluster to contract
and the nuclei to migrate towards the edge of the soma in addition to other
behaviors seen in isolated cells (Ishii et al., 2000). Further studies have
demonstrated a time course of cytoskeletal responses after an LPA signal. LPA
treatment causes an initial wave of actin depolymerization and loss of membrane
ruffling in neurons, followed by a second phase of actin polymerization involving
process retraction and rounding (Fukushima et al., 2000). In other cell types, the
responses vary. For example, in mouse skin fibroblasts and some cancer cell
lines, addition of LPA induces cell motility and migration (Hama et al., 2004). So
far, it is not clear how LPA mediates the different cytoskeletal effects in different cell types or how these responses relate to *in vivo* processes and functions.

**LPA may induce cell proliferation and survival pathways**

Studies in cell lines also demonstrate a critical role for LPA signaling in inducing cell proliferation and survival. LPA stimulates proliferation through LPA receptors in human retinal pigmented epithelial cells (Thoreson et al., 1997). These data are supported by gain-of-function analysis, when individual LPA receptors are overexpressed in RH7777 and B103 lines that lack endogenous receptors (Fukushima et al., 1998). In response to LPA, there is a robust increase in the rate of proliferation in these cells. Intracellular mediators of proliferation pathways, including ERK, are activated in response to LPA in several cell types (Fang et al., 2000; Goetzl et al., 1999; Koh et al., 1998). One issue that must be resolved is that proliferative responses and activation of the MAPK family member ERK are not seen in all cell types. In sciatic nerve Schwann cells that are incubated in serum-free media, addition of LPA results in cell survival, but this survival response is not due to an increase in proliferation (Weiner and Chun, 1999). Therefore, LPA signals appear to play only a protective response and do not lead to increase in cell number. Proliferation differences may be attributed to growth factor production in certain cell types after serum (rich in phospholipids) withdrawal (Steiner et al., 2002), but not a direct effect of LPA addition.
LPA is a survival signal in many cell types. Downstream activation of PI3K and Akt leads to a block in apoptosis. The survival response in Schwann cells treated with LPA is blocked by addition of pertussis toxin (PTX) and two PI3K inhibitors, wortmanin and LY294002, suggesting the involvement of $G_{i/o}$ proteins and PI3K. Moreover, survival responses are blunted by transfection of a kinase dead mutant of Akt. One possible mechanism for survival is based on data using T lymphoblastoma cells. In this cell type, LPA treatment decreases levels of the pro-apoptotic family member Bax without altering levels of other apoptosis mediators (Goetzl et al., 1999). Therefore, a common response to LPA does appear to be blockade of apoptosis and stimulation of survival pathways.

The activation of MAPK family members differs in several cell types. This protein family may play key roles in regulating cell proliferation, survival, or apoptosis. In rat cortical secondary astrocyte cultures, ERK is diphosphorylated, but this response is not seen in MEFs. On the other hand, LPA treatment activates JNK in both cell types. It is unclear how an LPA signal may activate specific MAPK family members and how activation of these proteins translates into specific behaviors. Although a general response to LPA seems to be cell survival, and in some cell types an increase in proliferation, there are few data suggesting how these responses are utilized in vivo or if there is a common pathway used in all cell types versus tissue and cell-specific responses. Determining how downstream responses are regulated in vivo may uncover how LPA signaling may be involved in pathophysiological states, such as
atherosclerosis and ovarian cancer (Goetzl et al., 2002), and manipulated by pharmacotherapy.

**In vivo roles of lipid receptors**

The first implication of lipid receptor function in development came from a mutation in the *Danio rerio* gene encoding the ortholog to S1P$_2$, *miles apart* (mil). In the mil mutant, there is a cardia bifida phenotype where the two heart primoridia form laterally and are patterned correctly, but never migrate and fuse in the midline to form a single, functional heart. The role for Mil is not in the migration of cardiomyocyte precursors, but in the underlying substrata over which these cells migrate. It is hypothesized that critical functions of Mil may be to mediate cell-cell contacts, act as a chemoattractant, and to regulate a proper environment for cardiomyocyte migration (Kupperman et al., 2000).

Targeted deletions of the LPA receptors have been made for three of the LPA receptors in mouse. The phenotype of the LPA$_1$ KO mouse includes craniofacial abnormalities, involving a reduced snout size and widely spaced eyes. X-ray analysis of the skull demonstrates reduced bone sizes, but all bones in the face are present. Also, these mice have reduced growth and a 30% reduction in body weight compared to wild-type littermates. These mice have decreased fat deposits, which may contribute to the size differences. During the first three weeks postpartum, there is 50% lethality, attributed to olfactory defects. The pups display normal rooting reflexes and search for the nipples, but cannot locate them. Consequently, little milk is present in the stomachs of the
pups. Finally, there is a small incidence of frontal hematomas (2.5%). There is increased apoptosis in Schwann cells surrounding the sciatic nerve, but this level of apoptosis is not enough to interfere with function, as reflexes and motor function are normal (Contos et al., 2000). Based on the many functions proposed by the in vitro data, it is surprising that more phenotypes are not detected in the knockouts. Moreover, there is no compensatory increase in the levels of other LPA and S1P receptors in this mouse, suggesting that any roles not uncovered here are due primarily to redundancy between receptors.

Targeted deletion of LPA$_2$ shows no phenotypic abnormalities compared to wild-type at gross and microscopic levels. Targeted deletion of both LPA$_1$ and LPA$_2$ produces no further defects beyond those seen with LPA$_1$ alone, although there is an increase in the number of frontal hematomas. Regardless, mice with the double deletion are viable and able to reproduce. Examination of neural phenotypes in the LPA$_2$ KO demonstrates that BrdU incorporation is normal and interkinetic nuclear migration occurs similarly to wild-type mice. The cortex is patterned properly and the thickness and size of the cortex is unaffected (Contos et al., 2002).

To study additional responses in the developing cortex, a culture model was developed. In this model, the cortex is isolated from developing mice, split into two, and one half is treated with LPA. LPA treatment of cortices increases the amount of cortical folding and this effect is attributed primarily to an increase in cell number in both the proliferative and post-mitotic zones. Cell density in these regions is normal. There is a decrease in end-labeling of DNA fragments
and caspase 3 activation (markers of apoptosis), suggesting that there is a substantial increase in the proliferating pool of neurons, although rates of proliferation are not changed in vivo, and that neurons are protected from apoptosis (Kingsbury et al., 2003).

Recently, a mouse with targeted deletion of LPA₃ was made. These mice are born normally, are of normal size, have no neurological deficits, and normal mendelian ratios are seen, similar to the LPA₂ deletion. However, females carrying the deletion in LPA₃ produce litters that are less than 50% of the size compared to wild-type. LPA₃ is expressed within the female reproductive system, and transcript levels vary based on cycling and pregnancy status. Defects are due to defective and delayed implantation leading to overcrowding within the uterus. Implantation sites are reduced within the uterus, and this is not due to a decrease in the number of blastocysts produced, but an increase in the frequency of blastocysts occupying the same implantation site. LPA is thought to control this behavior by regulating the production of prostaglandins E₂ and I₂ (Ye et al., 2005).

To determine individual roles for the LPA receptors, more assays must be developed to test different behaviors that these receptors may control. A recent report demonstrates the use of antisense oligonucleotides to downregulate the LPA receptors locally. Injection of LPA intrathecally into the region of the dorsal root ganglion (DRG) in the lumbar spinal cord causes thermal hyperalgesia (increase pain perception) and mechanical allodynia (pain in response to a stimulus that should normally not induce pain). Both responses are blocked by
injecting antisense nucleotides against LPA₁ prior to LPA injection or by injecting LPA into a mouse with targeted deletion of LPA₁. This effect is mediated through a Rho-ROCK dependent mechanism, since the response is blocked by exoenzyme C3 (ExoC3 – a Rho inhibitor) and Y27632 (a ROCK inhibitor). LPA injection leads to demyelination of the sciatic nerve, which is mimicked in cases of nerve transection. Finally, LPA induces expression of PKCγ, a sign of nerve injury and neuropathic pain, and the expression is blocked by ExoC3. By examining phenotypes under certain physiological and pathological conditions, such as nerve injury, it is possible that more roles will be uncovered in the single and double knockout animals. It is possible that several lipid receptors have to be targeted in mammals before substantial phenotypes are seen (Inoue et al., 2004).

**Studying roles of LPA receptors during early development**

The *in vitro* data on LPA signaling demonstrates that these receptors are able to activate a variety of downstream cascades in similar and differential manners, and that the responses are able to influence many cell processes including cell proliferation, survival, differentiation, and morphology. Receipt of an LPA signal is a general overall “survival” signal to a cell. This signal allows the cell to activate survival pathways while inhibiting apoptosis and to mobilize microfilaments into a rigid network that may protect the cell from physical forces. Work from zebrafish and mouse is beginning to delineate how these processes are utilized in an *in vivo* setting. However, many of the processes thought to
require LPA do not appear to be affected. Moreover, the mice with targeted deletions of LPA receptors are viable and fertile and there are no effects in early developmental processes that are apparent in these models. It is possible that a great deal of redundancy exists in mammals and only with triple and quadruple knockouts will roles of these lipids be determined.

*Xenopus* is a useful model system to study how these lipids function during early development. In *Xenopus*, only two LPA receptors and one S1P receptor have been identified (Lloyd et al., 2005). One of the LPA receptors is maternal, while all three are expressed zygotically by the time gastrulation commences. Since there is far less redundancy, it is easier to target each of these receptors. Additionally, early events in *Xenopus* are easily studied due to the size of the embryo and the ease with which manipulations and tissue dissections may be performed. Finally, since a fate map is available, reagents may be targeted to specific lineages and injected cells may be followed to analyze the role of these receptors in specific tissues.

**Hypothesis:**

The goal of this dissertation is to uncover roles for LPA receptors in early *Xenopus* development. In the first part, we examine the hypothesis that the two LPA receptors at the blastula stage act as a factor that may control the density of the cortical actin cytoskeleton in interphase blastomeres in the animal cap. In the second part, we examine the hypothesis that XLPA1 mediates cell survival and
cell proliferation signals in the anterior CNS and that this is critical for proper development.

References


CHAPTER II


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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.

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 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., 2002a). 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* XLPA₁ 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 orthologue 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; Fang et al., 2000). 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 LPA$_1$, LPA$_2$, LPA$_3$ (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 LPA$_1$ (and designated here as XLPA$_{1A}$ and XLPA$_{1B}$). 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 LPA$_2$ (designated here as XLPA$_2$) is expressed after the onset of zygotic transcription. No homolog of mammalian LPA$_3$ 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 XLPA$_1$ and XLPA$_2$ 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 using 1 µM progesterone and fertilized using the host transfer technique as reported previously (Holwill et al., 1987). Embryos were dejellied in a 2 % cysteine/ 0.1 x MMR solution (pH=7.8) and maintained in 0.1x MMR. Embryo stages cited are as described by Nieuwkoop and Faber (Nieuwkoop, 1967a). For mRNA and morpholino injections after fertilization, embryos were transferred into a 2% ficoll/ 0.5x MMR solution and injected into the animal cytoplasm of each blastomere at the 2-cell stage.

Analysis of the actin skeleton

Vitelline membranes were removed from stage 9 embryos in a 1x MMR solution on agarose dishes. Embryos were fixed for 30 minutes in FG fixative (3.7% formaldehyde/ 0.25% glutaraldehyde/ 0.2% 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 in 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 1x MMR and 0.4% lipid-free BSA was added to all solutions as a carrier. After caps were cut, they were incubated for ten minutes in a lipid or control solution before analysis of cortical actin.

**Oligonucleotides**

Twelve antisense oligonucleotides complementary to both XLPA$_{1A}$ and XLPA$_{1B}$ mRNA were tested for their ability to deplete the maternal messages by injecting into the marginal zone of oocytes, incubating for 24 hours at 18 °C, and assaying for mRNA depletion using RT-PCR. 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 elected for use is as follows (where asterisks represent phosphorothioate linkages):

**LPA$_1$-10MP:** 5’ T*C*A*TTGTAGTAGCAC*T*G*G 3’

Morpholino oligonucleotides were designed that targeted both XLPA$_{1A}$ and XLPA$_{1B}$ or XLPA$_{2}$. These were resuspended in sterile, filtered water and injected at doses of 10-40 ng into either oocytes or embryos.

**XLPA$_{1A}$ and 1B MO:** 5’ TTCACTTCAGATGTCAGTCATGCTG 3’

**XLPA$_2$ MO:** 5’ ACCTCCAATGTTACAGCGCAGCCTC 3’
RNA Constructs

Clones encoding both *X. tropicalis* XLPA$_1$ and XLPA$_2$ were identified by blasting the murine sequences for LPA$_1$ against *X. tropicalis* cDNA libraries at the Sanger Institute site (http://www.sanger.ac.uk/). The following clones for XLPA$_1$ (TNeu092p02) and XLPA$_2$ (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 Apal. *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.

RTPCR

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 DNase I treated. 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 was normalized to either ornithine decarboxylase (ODC) 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 and look for the formation of primer dimers. Primer pairs that were used are as follows:
XLPA₁ – F: 5’ CTTGGAGTCCCGTGTGTTTT 3’  
R: 5’ TGGCTGCAGAAGTCTGTGAC 3’  
XLPA₂ – F: 5’ TTCTTCTGCAACAGGGGTTC 3’  
R: 5’ GGGCCTCACTCCAACTGT 3’  

ODC – F: 5’ GCC ATT GTG AAG ACT CTC TCC ATT C 3’  
R: 5’ TTC GGG TGA TTC CTT GCC AC 3’  

Plakoglobin – F: 5’ GCT CGC TGT ACA ACC AGC ATT C 3’  
R: 5’ GTA GTT CCT CAT GAT CTG AAC C 3’  

**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 three minutes (Snape et al., 1987). Dissociated cells were transferred into 1x calcium/magnesium-free MMR on a 1% agarose dish. After one hour, cells were transferred into 0.1-1 µM LPA in calcium/magnesium-free MMR in glass dishes for five minutes, or allowed to reassociate in 1x MMR. Cells were removed from the LPA solutions and maintained in 1x calcium-magnesium free MMR for different time intervals before fixation. Cells were fixed for 4 minutes in FG fix, washed with 1x PBS+0.1% Tween-20, and stained with Alexa 488-phalloidin. To determine if calcium or magnesium affected the actin cytoskeleton of dissociated cells, the cells were transferred back into 1x 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 5,000 µm² area for at least 15 dissociated cells in each group. For animal caps, the mean intensity was recorded over a 0.62 mm² area for gain-of-function experiments and a 1,000 µm² area with the low threshold set to 100. The mean intensities were averaged and are reported as mean ± S.E.M. The Student’s t-test was used to determine significance and p < 0.05 was considered 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 ten minutes before fixation. Fig.1 shows a dissecting microscope view of caps fixed before isolation (Fig. 1A), and after ten 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 and D, and see Kofron et al. 2002). Cells extended occasional filopodia (arrowed in Fig. 1D). In caps that were allowed to heal for ten minutes before fixation, actin-rich purse-strings formed around the margins of the caps (arrowed in Fig.
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 and 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 calcium/magnesium free saline, we compared dissociated cells that had been cultured in calcium/magnesium-free MMR before fixation with those that were transferred into 1x 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 1x MMR resulted in re-assembly 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 ten 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 caps 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 to 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 (data 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*: XLPA<sub>1A</sub> and XLPA<sub>1B</sub> (Kimura et al., 2001). These both show 90% homology to the human LPA<sub>1</sub> 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 orthologues of the mammalian LPA<sub>1</sub> receptor. The sequences for murine LPA<sub>2</sub> and LPA<sub>3</sub> 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<sub>2</sub>. These were obtained and sequenced and found to encode the same mRNA. Since TNeu013j17 contained the full coding sequence, this was used for experiments described here. The full sequence of *X. tropicalis* XLPA<sub>2</sub> mRNA was deposited into GenBank as accession number AY652941.

The predicted protein was found to be 62% identical and 16% similar to mouse LPA<sub>2</sub> at the protein level and thus was designated *X. tropicalis* XLPA<sub>2</sub>. 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<sub>2</sub> is most divergent from the mammalian orthologues 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<sub>2</sub> 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 orthologues 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 RT-PCR. 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. This experiment was repeated in three independent experiments and the same result was obtained each time.

**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 2-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\textsubscript{1} or XLPA\textsubscript{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\textsuperscript{2} area from 1133 ± 177 to 1372 ± 302 or 1610 ± 348 for XLPA\textsubscript{1} and XLPA\textsubscript{2}, respectively (Fig. 4B). All data represents four independent experiments with five caps per group in each experiment. 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\textsubscript{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\textsubscript{1} mRNA using antisense-oligodeoxynucleotides. Twelve oligos, each complementary to both XLPA\textsubscript{1A} and XLPA\textsubscript{1B}, were synthesized and tested for their ability to deplete both XLPA\textsubscript{1} 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). XLPA$_1$ mRNA levels were reduced to 16% of control levels at the 2-cell stage (Fig. 7A). At stage 10, XLPA$_1$ 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 to control embryos. Animal caps from depleted embryos, and the bases from which they were excised, healed more slowly than controls (Fig. 7B,C). At higher magnification, actin filament bundles in the cell cortices were dramatically reduced in density, compared with controls (Fig. 7D). The formation of actin-rich filopodia was unaffected in these embryos. Neither the overexpression nor depletion of LPA receptors affected the number or size of contractile rings seen in dividing cells. The caps contained the normal number of cells and there was no evidence of undividing cells in these embryos (data not shown). The effects of the thioate oligo were reversed (Fig. 7C) by depleting XLPA$_1$, fertilizing the oocytes by host transfer, and injecting 400 pg of XLPA$_1$ mRNA at the 2-cell stage, by which time the antisense oligo had degraded (Raats et al., 1997). The average mean
intensity of phalloidin staining decreased from 1532 ± 395 to 978 ± 202 and 877 ± 191 for the 7.5 ng and 10 ng dose of XLPA1-10MP, respectively (Fig. 4C). Data are representative of three independent experiments with 5 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 XLPA1-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 5 animal caps per group.

Depletion of XLPA$_2$ 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 XLPA$_1$ (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 morpholinos reduced the average mean intensity of phalloidin staining from 1122 ± 87 to 1002 ± 59 (Fig. 4D). These data suggest that after the mid-blastula stage, the combined levels of the two LPA receptors are necessary to maintain the pattern and density of cortical actin in the embryo. In contrast to removal of only the maternal XLPA$_1$ mRNA, embryos that were injected with XLPA$_1$ or XLPA$_2$ 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), XLPA$_1$ or XLPA$_2$-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 which had been dissociated at the mid-blastula stage and kept apart for one hour, and cells kept apart for one 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 to 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 5,000 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 both mRNAs at the 2-cell stage and analyzed the actin skeleton at stage 9. Overexpression of RhoA-N19 alone resulted in a loss of purse-strings (Arrowed 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 (data not shown). When XLPA2 and RhoA-N19 were injected together, the Rho-N19 blocked the effects 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 purse 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 coinjected 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 behaviors, including proliferation, survival, motility, cell shape and differentiation (Anliker and Chun, 2004; Contos et al., 2002; Fukushima et al., 2002; Tigyı,
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. This suggests 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 XLPA₁ and XLPA₂ 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. Also, 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 critical 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$_1$. This was most likely due to the onset of XLPA$_1$ and XLPA$_2$ production after the MBT. Redundancy also exists between murine LPA receptors. The $lpa_2^{(-/-)}$ mouse showed no obvious gross or histological phenotype and the $lpa_1^{(-/-)}lpa_2^{(-/-)}$ mouse only showed an increase in frontal hematomas compared to the $lpa_1^{(-/-)}$ 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 $lpa_1^{(-/-)}lpa_2^{(-/-)}$ 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 XLPA$_1$ and XLPA$_2$ 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 $G_{12/13}$ 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 $G_\alpha i/o$ mediated pathway to exert its effects (Van Leeuwen et al., 2003). Although we have not determined which G proteins are utilized 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, 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 \( lpa_1^{-/-}lpa_2^{-/-} \) mouse, normal wound healing was observed compared to control mice, but this may also be 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 actinomyosin 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


Figure legends

Fig. 1. F-actin-containing structures in early *Xenopus* embryos. (A) Animal caps removed from late blastulae (St. 9) and photographed immediately after excision (0 minutes) or 10 minutes after healing (B). Scale bar: 125 µm. (C and D) Low and high magnification views of F-actin staining of the actin network of a late blastula animal cap. Scale bar: C=20 µm, D=5 µm. (E) F-actin staining of an animal cap that had been removed from the embryo and allowed to heal for 10 minutes. Arrow indicates the purse-string. Inset shows a high-power view of the inner cells of the cap. Scale bar: 50 µm. (F) High magnification (126x) image of area outlined in (E) showing the contractile ring and coarser actin filament network in a dividing cell. Scale bar: 5 µm.

Fig. 2. Dissociation of blastula stage animal caps causes a reduction in the density of cortical actin, which is rescued by reaggregation of the cells. Low- (A) and high- (B) magnification images of the F-actin network *in situ* in caps before disaggregation. (C) The coarser F-actin network found in single cells 45 minutes after dissociation. (D) Reassociation of the animal cap cells caused the cortical actin network to return to normal levels. Scale bars: 20 µm in A and 5 µm in B-D.

Fig. 3. Addition of LPA to animal caps causes an increase in cortical actin and more rapid wound-healing. (A) Experimental design. Animal caps were excised
from late blastulae, incubated for 10 minutes in either control or LPA solutions, fixed and stained with Alexa-488 phalloidin to visualize F-actin. Caps incubated in 1 μM LPA (C) had increased cortical actin and more rapid wound-healing, compared to control caps (B). (D and E) Higher magnification images of control and 1 μM LPA-treated (C) caps. The increased cortical actin and surface projections caused by LPA are shown. (F) Incubation of animal caps in 5 μM phosphatidic acid for 10 minutes did not increase cortical actin or expedite wound-healing. Scale bars: 50 μm in B,C,F; 10 μm in D,E.

**Fig. 4.** Quantitation of changes in the intensity of phalloidin staining. There is a significant increase in the intensity of phalloidin staining if caps are treated with LPA (A) or if either LPA receptor is overexpressed in caps (B), but not with treatment of the related phospholipid phosphatidic acid (A). (C, D) There is a significant decrease in the intensity of phalloidin staining by targeting the mRNA with either a phosphorothioate oligo or morpholino oligonucleotides. Asterisks indicate significance at p < 0.05. All data are representative of a single experiment and all experiments were repeated at least three times with five caps per group.

**Fig. 5.** Identification of a *Xenopus tropicalis* LPA receptor that is similar to mammalian LPA₂. (A) Alignment of *X. tropicalis* XLPA₂ with mouse and human LPA₂. Putative transmembrane domains (TMD) are indicated above the sequence. Residues important for LPA binding and specificity in other species
are highlighted in red. *X. tropicalis XLPA*₂ is 62% identical and 16% similar to murine LPA₂. (B) Expression using real-time RTPCR of XLPA₁ and XLPA₂ mRNA during early development. Each bar represents the amount of mRNA present, normalized to the loading control ornithine decarboxylase. As reported previously, XLPA₁ is abundant maternally and continues to be expressed at a low level throughout development. Expression of XLPA₂ begins at the mid-blastula stage and continues to at least stage 45. St.2, 2-cell stage; St.9, late blastula; St.10, early gastrula; St.12, late gastrula; St.15, early neurula; St.20, late neurula; St.35, tail-bud; St.45, swimming tadpole.

**Fig. 6.** Overexpression of either *X. tropicalis* XLPA₁ or XLPA₂ mRNA causes an increase in cortical actin and more rapid wound healing, mimicking LPA treatment. (A) The bases (after excision of animal caps) of embryos injected with 400 pg of XLPA₁ elongated along the animal-vegetal axis after the vitelline membrane was removed and were more rigid than control embryos. These bases healed faster than controls. Scale bar: 350 \( \mu \text{m} \). (B) Bases from embryos injected with XLPA₂ mRNA showed a similar phenotype as bases from XLPA₁-injected embryos. Scale bar: 450 \( \mu \text{m} \). (C) Animal caps from XLPA₂-injected embryos rounded up faster than control caps after 10 minutes of healing. Scale bar: 125 \( \mu \text{m} \). Injection of either XLPA₁ (D) or XLPA₂ (E) mRNA increased the levels of cortical actin in the animal cap cells and in the purse-string compared with controls. Scale bar: 50 \( \mu \text{m} \).
**Fig. 7.** Maternal XLPA<sub>1</sub> is required *in vivo* for modulating actin assembly in early *Xenopus* development. (A) Injection of 10 ng of an antisense phosphorothioate oligodeoxynucleotide (LPA<sub>1</sub>-10MP) into oocytes caused a reduction in XLPA<sub>1</sub> mRNA to 16% of control levels after fertilization. At the beginning of gastrulation (stage 10), this reduction was one-third of control levels. (B) Bases from embryos depleted of maternal XLPA<sub>1</sub> healed more slowly than controls. Scale bar: 280 µm. (C) Animal caps from depleted embryos (middle) showed decreased levels of F-actin in the animal cap and in the purse-string after wounding. These caps were larger than controls (left) due to slower wound-healing. The effects of the oligo were rescued by injecting *X. tropicalis* XLPA<sub>1</sub> mRNA back into fertilized eggs that had been depleted of XLPA<sub>1</sub> (right). This demonstrates that the effects are specific to depletion of XLPA<sub>1</sub>. Scale bars: 50 µm. (D) High magnification (63x) image of the cortical actin network in control (left) and XLPA<sub>1</sub>-depleted caps (right) after 10 minutes of healing. The density of actin filaments is greatly reduced in the depleted caps compared to controls. Scale bars: 10 µm. (E) Depletion of the maternal stores of XLPA<sub>1</sub> does not lead to long-term developmental defects. Embryos were able to gastrulate (left) and develop to tail-bud stages (right). Control embryos are red and XLPA<sub>1</sub>-depleted are brown. Scale bars: 350 µm (left) and 750 µm (right).

**Fig. 8.** Dominant negative forms of RhoA and Rac1 block the overexpression effects of XLPA<sub>2</sub>. (A) Low power magnification (Scale bar - 20 µm). (Left panels) A control cap (upper) and a cap injected with 100 pg of XLPA<sub>2</sub> mRNA (lower) into
each cell at the 2-cell stage. (upper middle) Overexpression of RhoA-N19 blocks purse-string formation and delays wound healing with no change on cortical actin. (lower middle) The overexpression effects of XLPA₂ on wound healing are blocked by RhoA-N19, but not the increase in cortical actin. (Right panels) Rac-N17 also prevents purse-string assembly and reduces the amount of cortical actin (upper) and blocks the effects of XLPA₂ (lower). (B) High power magnification. Scale bar: 5 µm. (upper left) The cellular network in a cap injected with a low dose of XLPA₂ mRNA. (lower left) Injection of RhoA-N19 results in an increase in cellular processes and (upper middle) prevents the formation of an actin purse-string (Arrow). (upper right) Cells in caps injected with both XLPA₂ and RhoA-N19 still have cell processes similar to RhoA-N19 alone and no purse-string. (lower middle) Rac-N17 caused a decrease in the amount of cortical actin, decreased the number of cell processes, and caused the cells to become rounded. (lower right) When coinjected with XLPA₂, Rac-N17 blocks the increases in cortical actin and formation of a rigid network, but cell processes are still evident.

**Fig. 9.** Zygotic expression of LPA receptors is necessary to maintain the density of the actin cytoskeleton in late blastula stage and for normal *Xenopus* development. (A) Depletion of XLPA₁ by injection of 40 ng of a morpholino oligonucleotide (MO) into oocytes or eggs caused a reduction in the amount of cortical actin at the late blastula stage. Scale bar: 50 µm. (B) Injection of 30 ng of LPA₂ MO into eggs reduced the density of actin by the same amount as did the
LPA₁ MO. Injection of 15 ng of both MO simultaneously caused a reduction in actin compared to the higher doses of the individual MOs. Scale bar: 10 µm. (C) Embryos depleted of either LPA receptor or both exhibited delays in gastrulation and closure of the blastopore. Scale bar: 280 µm. (D) An array of defects was seen in depleted embryos during the neurula stages from reduction in the size of neural folds to failure of the neural folds to form. Scale bar: 350 µm. (E) At late tail-bud stages (St. 37-8), embryos displayed pleiotropic defects including a shortened anterior-posterior axis, reduced heads, and an open neural tube. Scale bar: 450 µm.

**Fig. 10.** Addition of LPA to dissociated cells increases the density of the cortical actin network. (A) Cells treated for 5 minutes with 1 µM LPA show an increase in the density of cortical actin compared to control cells. Scale bar: 5 µm. (B) Quantitation of the levels of cortical actin in individual dissociated cells measured by pixel intensity of Alexa 488-phalloidin fluorescence. Bars 1 and 6 represent dissociated cells, not treated with LPA, at the beginning and end of the experiment respectively. Bars 2 - 5 represent cells treated for 5 minutes with LPA, which was then washed out, and cells fixed 0, 15, 30, and 45 minutes later. Asterisks represent a significant difference (p < 0.05) compared to control.
Figure 1
Figure 3

A. Cut caps at stage 9
   Allow to heal for 10’ in LPA
   Fix caps and stain for f-actin

B. Control
C. 1 uM LPA

D. Control
E. 1 uM LPA
F. 5 uM PA
FIGURE 4
Figure 5

A

T1pa2
Mouse_LPA2
Human_LPA2

TMDII

TMDIII

TMDIV

TMDV

TMDVI

TMDVII

B

Expression of XLPA1

Expression of XLPA2
Figure 10
CHAPTER III

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Lysophosphatidic acid signaling through the receptor XLPA₁ is necessary for cell survival and proliferation in the early central nervous system of *Xenopus laevis*

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Summary

Lysophosphatidic acid (LPA) is a signaling ligand that activates a family of G-protein coupled receptors (GPCR). In vitro, it affects many of the cellular processes that are essential to normal organogenesis. However, its role in early development in vivo has been difficult to establish, due to potential redundancy between the large number of receptors and ligands in mammalian models. We have used early postgastrula development in *Xenopus laevis* as a model, since there is less receptor redundancy and a defined fate map is available to target reagents to discrete post-gastrulation cell lineages. We find that loss-of-function of the XLPA<sub>1</sub> receptor in the developing central nervous system (CNS) results in a dramatic loss of anterior, but not posterior, CNS structures. Analysis of molecular markers shows that specification of the neural plate and tube occurs normally, but is followed by increased apoptosis and reduced proliferation in the anterior, but not posterior CNS. The receptor XLPA<sub>2</sub> is not essential for formation of the anterior CNS, and loss of XLPA<sub>1</sub> does not cause loss of epidermis. These data show for the first time that LPA signaling is essential for cell survival and proliferation in the anterior CNS, and that functions of its receptors diverge as the early embryonic cell lineages form in vertebrates.

Introduction

Extracellular LPA is a signaling ligand that activates a family of G protein-coupled receptors. In mammals, it activates the closely related LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub> receptors (previously known as the Edg receptors) and a more distantly related LPA<sub>4</sub> receptor ((Anliker and Chun, 2004). In *Xenopus*, only two LPA receptors
have so far been identified, XLPA₁ and XLPA₂, most closely related to the mammalian LPA₁ and LPA₂ respectively (Kimura et al., 2001; Tao et al., 2005). Members of the Edg receptor family have not so far been identified in invertebrate model systems, although there is abundant evidence that lipid signaling is important in the development of both *Drosophila* and *C. elegans* (Moore et al., 1998; Renault et al., 2002; Sano et al., 2005; Starz-Gaiano et al., 2001).

In vitro, LPA has dramatic effects on cell proliferation, cell survival, cell morphology, and gene expression. These pleiotropic effects are due to selective activation of G-proteins by the different LPA GPCR’s. LPA₁ and LPA₂ activate $G_{12/13}$, $G_{i/o}$, and $G_q$; LPA₃ activates $G_{i/o}$ and $G_q$; and LPA₄ activates $G_s$ (Reviewed in Anliker and Chun, 2004). These in turn activate downstream pathways leading to cytoskeletal changes, cell proliferation, survival, gene expression, and cell differentiation. These pathways have been confirmed using embryonic fibroblasts cultured from mouse embryos carrying targeted mutations in the LPA receptors (Contos et al., 2002).

These aspects of cell behavior are essential components of normal morphogenesis. In vertebrates, organ rudiments form after gastrulation by region and tissue-specific changes in cell proliferation, migration, and survival. However, despite their dramatic effects on these processes in culture, there is surprisingly little data on the roles of LPA during early organogenesis in vertebrates.
Targeted mutagenesis of the LPA$_1$ receptor in mice causes nervous system defects, frontal hematomas, and 50% neonatal lethality that is attributed primarily to olfactory system defects and impaired suckling behavior in the pups. LPA$_1$-null pups have a 30% reduction in body weight compared to the control littermates and craniofacial abnormalities. There is an increase in Schwann cell apoptosis in the sciatic nerves, which is not sufficient to cause loss of motor functions (Contos et al., 2000). These mice also mimic some aspects of psychiatric disease, having alterations in CNS amino acid levels and serotonin metabolism (Harrison et al., 2003). LPA$_2$ targeting causes no abnormalities at the gross or microscopic levels and the mice are viable. Targeting of both receptors causes similar defects to that of LPA$_1$ only, with a slight increase in the incidence of frontal hematomas (Contos et al., 2002). Loss-of-function studies have also been carried out using antisense oligonucleotides injected intrathecally into the dorsal root ganglion at lumbar levels. Depletion of LPA$_1$ blocks thermal hyperalgesia and mechanical allodynia responses, induced either by injection of LPA or by nerve ligation (Inoue et al., 2004). Finally, in a mouse cortical hemisphere culture system, LPA treatment induces cortical folding due to an increase in cell number, promotes terminal cell mitosis, and inhibits cell death. These responses were blocked in hemispheres explanted from embryos null for LPA$_1$ and LPA$_2$. However, cortical development was not affected by targeting of either or both of these receptors in vivo (Contos et al., 2002; Kingsbury et al., 2003). Targeting of the LPA$_3$ receptor has the interesting effect of altering the spacing of implantation of blastulae along the female reproductive tract (Ye et al., 2005).
Targeting of the LPA receptors in mouse has not so far revealed any serious defects in early organogenesis. The reasons for this are not clear. It may be due to redundancy between receptors, or ligands, during early development, or because LPA does not have essential functions during early morphogenesis. Data from zebrafish, however, suggests that the related phospholipid, sphingosine-1-phosphate (S1P), is important in early organogenesis (Kupperman et al., 2000). Embryos with a mutation in *miles apart (mil)*, a mutant of the S1P receptor S1P2, have a cardia bifida phenotype due to defective migration of cardiac precursors over their cellular substrata. It is suggested that mil protein is required in the substrata to regulate this migratory process.

We have previously shown that the early *Xenopus* embryo offers a useful model system for the analysis of LPA signaling during pre-gastrulation development (Tao et al., 2005). Two LPA receptors, corresponding to mammalian LPA1 and LPA2, have been identified in the early embryo. XLPA1 mRNA is expressed both in the oocyte and during embryogenesis, whilst XLPA2 mRNA expression commences at the mid-blastula stage. Both gain-of-function, by mRNA injection, and loss-of-function, by injection of morpholino (MO) antisense deoxyoligonucleotides (oligos) into the oocyte, identify an essential function of the two XLPA receptors in controlling the density of the cortical actin network (Lloyd et al. 2005).
In this paper, we show that XLPA₁ is also required during post-gastrula stages for the normal development of the anterior CNS. Targeting of the XLPA₁ MO to a single blastomere giving rise to the CNS on one side of the embryo revealed that XLPA₁ is required for survival and proliferation, but not the induction of early neural progenitor cells. Rescue experiments showed that this property is specific for XLPA₁, which cannot be replaced by XLPA₂. Furthermore, XLPA₁ does not have the same essential function in the spinal cord and epidermis. These data show for the first time that XLPA₁ signaling is essential during early CNS development in vertebrates, and that the functions of the LPA receptors diverge as early cell lineages form in the vertebrate embryo.

Materials and Methods

Embryos and microinjections

Embryos were obtained by in vitro fertilisation as described previously (Tao et al., 2005), dejellied in 2% cysteine/0.1X MMR (pH = 7.8), maintained in 0.1X MMR, and staged according to (Nieuwkoop, 1967b). For injections, embryos were transferred into 2% ficoll/0.5X MMR. For loss-of-function experiments, 20 – 40 ng of the XLPA₁ or XLPA₂ morpholino (MO) (Gene tools) was coinjected with 6 ng of the lineage tracer rhodamine lysine dextran (RLDX) into a single dorsal, animal blastomere at the 8-cell stage. Rescue experiments were performed by injecting 30 ng of the XLPA₁ MO and 6 ng of RLDX as described above. The injected blastomere was then reinjected with 50 pg of a morpholino-resistant mRNA encoding XLPA₁ or 50 – 200 pg of XLPA₂ RNA along with 300 pg of GFP.
mRNA as a lineage tracer. Alternatively, the XLPA1 MO was coinjected with both the XLPA1 or XLPA2 mRNA and RLDX.

**XLPA1 MO:** 5' TTCACTTCAGATGTCAGTCATGCTG 3'

**XLPA2 MO:** 5' ACCTCCAATGTACAGCGAGCCCTC 3'

**Real-time RTPCR**

For quantitative RTPCR on explants, animal caps, equators, and bases were isolated from 10 embryos at stage 9 and cultured until stage 19. RNA and cDNA were prepared as described previously (Zhang et al., 1998), with the modification that RNA was quantitated and 0.7 µg was used for each cDNA synthesis. RNA was also isolated from two whole embryos (WE) cultured alongside the explants. Real-time RTPCR was carried out using the Light Cycler System (Roche) and samples were normalized to the loading control ornithine decarboxylase (ODC). Primers used were described previously in XMMR:

[http://www.xenbase.org/xmmr/Marker_pages/primers.html](http://www.xenbase.org/xmmr/Marker_pages/primers.html) and (Heasman et al., 2000; Sasai et al., 1996; Sun et al., 1999; Tao et al., 2005). Experiments were carried out in triplicate in different batches of embryos with similar results.

**Analysis of the actin skeleton**

Vitelline membranes were removed from mid to late neurulae (stage 15, 18). Embryos were fixed for 20 minutes in FG fixative, 3.7% formaldehyde/0.25% glutaraldehyde/0.2% Triton X-100 in PIPES buffer (Gard et al., 1997), and washed in 1x phosphate buffered saline + 0.2% Triton-X 100 (PBST). Embryos were stained for F-actin with Alexa 488-phalloidin (Molecular Probes) and
imaged on the Zeiss confocal microscope as described previously (Kofron et al., 2002; Tao et al., 2005).

**Plasmids and mRNA transcription**

For both XLPA\(_1\) and XLPA\(_2\), the coding sequence was PCR-amplified using primers containing BamHI restriction sites. These inserts were digested with BamHI and subcloned into the BamHI site of pCS107. Plasmids were linearised with Asp718 and in vitro transcription for full length mRNAs was performed using the SP6 mMessage Machine Kit (Ambion). Following transcription, samples were treated for 15 minutes with DNase I, purified by phenol:chloroform extraction, and resuspended in sterile, filtered water. These mRNAs are resistant to inhibition by the respective morpholinos.

**In situ hybridization and histology**

Before whole-mount in situ hybridization, embryos were sorted based on location of RLDX to verify injection targeting. Embryos were fixed for 1.5 hours in MEMFA (0.1 M MOPS (pH = 7.4), 2 mM EGTA, 1 mM MgSO\(_4\), and 4% formaldehyde) at room temperature and processed for in situ hybridization as described previously (Harland, 1991) using BM purple (Roche) as a substrate. For histology, embryos were dehydrated after fixation, transferred into xylene, embedded in paraplast (Fisher) and cut at 12 \(\mu\)m sections on a Leica microtome. In situ probes for XLpa\(_1\) and XLpa\(_2\) were created by linearising XLPA\(_1\)-CS107 or XLPA\(_2\)-CS107 with NotI (sense) or HindIII (antisense). Transcription for XLpa\(_1\) and XLpa\(_2\) was performed using SP6 for a sense probe and T7 for an antisense probe. The following
probes were synthesized and used as described previously: Sox2 (Mizuseki et al., 1998), Pax6 (Hirsch and Harris, 1997), Xcp1 (Knecht et al., 1995), en-2 (Hemmati-Brivanlou et al., 1991), krox20 (Macrae et al., 1996), Xotx2 (Pannese et al., 1995), Neural-tubulin (Chitnis et al., 1995), and Nkx2.1 (Small et al., 2000).

**HP3 Staining and TUNEL**

Immunostaining for α-phospho-histone H3 (HP3) staining was performed with a monoclonal antibody directed against phosphorylated Ser10 (Upstate Biotechnology clone 3H10) at dilution of 1:1000. Cy5-Goat α-mouse IgG (Jackson ImmunoResearch) was used as a secondary antibody at 1:300. Sytox green (Invitrogen) was diluted to a concentration of 5 µM into the first wash after the secondary antibody and incubated for 10 minutes to label nuclei in sections. For analysis of whole-mount staining, embryos were dehydrated in methanol and transferred into Murray’s clear (2:1 benzoate/ benzyl alcohol) for imaging on the confocal microscope. Whole-mount TUNEL staining was performed as described (Hensey and Gautier, 1998) with the following modifications: after the TUNEL reaction was performed, embryos were incubated overnight with a 1:1000 dilution of a mouse monoclonal α-digoxigenin in block (Roche). Embryos were washed 6 times each for 20 minutes at room temperature, blocked, and incubated in a 1:200 dilution of Cy5-goat α-mouse (Jackson ImmunoResearch). TUNEL staining was visualized using confocal microscopy.
Results

LPA Receptors are widely expressed in the post-gastrula *Xenopus* embryo. Previously, we showed that both XLPA₁ and XLPA₂ receptors are expressed after the onset of zygotic transcription in *Xenopus* embryos. XLPA₁ is also expressed maternally. When both receptors are targeted, using antisense morpholino oligos (MO's), from the oocyte stage, an essential role in cortical actin assembly at the blastula stage was revealed. When these embryos were allowed to continue development, severe post-gastrula developmental defects were also found, suggesting either later essential roles for these receptors, or pleiotropic effects resulting from an earlier or continuing defect in actin assembly (Tao et al., 2005).

To determine where the receptors are expressed after gastrulation, we first dissected late blastula stage embryos into three components: animal caps (which form only epidermal tissue in culture), equators (which form derivatives of all germ layers, but primarily mesoderm in culture) and bases (which form only endodermal tissue in culture) and analyzed gene expression. Quantitative real-time RTPCR showed that XLPA₁ and XLPA₂ mRNAs were present in all three types of explant (Fig. 1A, middle panel). Markers for epidermis (*Epidermal keratin*), mesoderm (*Xbra*), and endoderm (*endodermin*) mRNAs were found to be enriched in caps, equators, and bases, respectively, thus confirming normal differentiation of these explants in culture (Fig. 1A, right panel). Second, we performed in situ hybridization. At the onset of neurulation, both XLPA₁ and
XLPA2 mRNAs were detected throughout the developing embryos, with highest levels in the CNS. Both receptors continued to be expressed throughout the CNS during neurulation (Fig. 1B-D). At tailbud stages (stage 28-30), XLPA1 and XLPA2 were still expressed at high levels in the CNS. XLPA1 was also enriched in the branchial arches and otic vesicle (Fig. 1E-F). Sectioning of the embryos showed that the mRNA was also enriched segmentally in the somites (Fig. 1G). XLPA2 was highly expressed in the cement gland, developing eye, forebrain, and spinal cord (Fig. 1E). In situ hybridization and quantitative real-time RT PCR were repeated in three independent experiments.

**XLPA1, but not XLPA2, is required for proper eye and anterior nervous system formation**

Since the XLPA1 and XLPA2 transcripts are expressed highly throughout the developing nervous system, we tested the possibility that they are required for its normal morphogenesis, making use of the *Xenopus* fate map (Moody and Kline, 1990). We targeted either the XLPA1 or XLPA2 morpholino, together with the lineage tracer RLDX, into a single dorsal, animal blastomere at the 8-cell stage. Descendants of this blastomere give rise to the entire CNS on one side of the body, as well as minor contributions to head somites and mesenchyme, branchial arches, and anterior foregut. The distribution of RLDX at Stage 15 was used to identify and select embryos with correct targeting of the MO (Fig. 2A,B). When analyzed at tadpole stages, embryos injected with 20 – 40 ng of the XLPA1 morpholino showed a dose-dependent loss of eye structures on the injected side (Fig. 2C,D,L). Embryos were classified into three groups: 1. normal, 2. mild -
embryos containing 50 – 99% of the normal eye diameter, and 3. severe - embryos containing < 50% of the normal eye diameter. At a MO dose of 40 ng, 78% of embryos had a severe phenotype and 23% of this group showed a complete absence of eye structures on the injected side (Fig. 2L). This phenotype could be rescued, either by subsequent injection of a MO-resistant XLPA1 mRNA (50pg) and GFP mRNA (300pg) into the same blastomere, or by simultaneous injection of the MO and the rescuing mRNA. Fig. 2F,G,L show and example of the first method. The XLPA₁ mRNA rescued the loss of eye phenotype (Fig. 2F,G,L) with a significant increase in the normal group (from 6% in the MO-injected, to 21% in the rescued group) and a significant decrease in the severe group (from 62% to 9% respectively; p < 0.05). Overexpression of the XLPA₁ mRNA at doses from 25 – 150 pg did not cause abnormalities (Fig. 2E,I,L).

Histological sections of embryos in the severe group at the early tadpole stage (stage 38) revealed that most of the anterior CNS on the injected side was missing, with cellular debris visible in the forebrain (Fig. 2I – arrow indicates injected side). Most of the eye had also degenerated on the injected side and no eye patterning was apparent. Epidermal cells in the same sections carrying the lineage marker, and thus also injected with the MO, did not degenerate and there were no breeches in the epidermal layer. Since the injections contribute to the entire CNS on the injected side, the spinal cord was also examined. Loss of XLPA₁ at the level of the hindbrain resulted in deterioration of the neural tube at this level also (Fig. 2J, K-a); however, the spinal cords (posterior CNS) were
normal in these embryos, as were labeled cells in the somites, notochord, and gastrointestinal tract (Fig. 2K-b). Injection of XLPA2 MO at doses of 20 – 40 ng (n = 30 per group) did not cause obvious developmental defects in any part of the CNS (Fig. 2H). These embryos formed normal forebrain and eye structures. This shows, together with the mRNA rescue experiments, that the defects seen were specific to the loss of the XLPA1 receptor and not due to a non-specific toxicity. Injections for both the XLPA1 and XLPA2 MO were repeated in four independent experiments with similar results.

**XLPA1 is specifically required in the early CNS**

In previous work, we showed that both XLPA1 and XLPA2 regulate the actin cytoskeleton at the blastula stage and that depletion of either XLPA1 or XLPA2, or both receptors, caused similar actin defects (Tao et al., 2005). Additionally, the actin phenotype seen in an XLPA1-depletion could be rescued by reintroduction of mRNA encoding XLPA2 and vice versa. To test whether XLPA2 could rescue the effects of XLPA1 depletion, XLPA2 mRNA was coinjected at doses of 50 – 200 pg with the XLPA1 MO and RLDX into a single dorsal, animal blastomere. Embryos injected with XLPA2 mRNA and RLDX only showed no defects in eye or CNS development (Fig. 3). There were no significant differences in embryos injected with either the XLPA1 MO alone, or together with XLPA2 mRNA (55% and 40% of embryos in the severe group, respectively - Fig. 3). These data demonstrate that XLPA2 is not able to compensate for XLPA1...
function at the neurula stage. These findings were obtained in three independent experiments.

**Defects caused by loss of XLPA<sub>1</sub> are not a result of reduced actin density**

During the blastula stage, LPA signaling through XLPA<sub>1</sub> and XLPA<sub>2</sub> is necessary to maintain a dense cortical actin network in interphase blastomeres of the *Xenopus* embryo. It is therefore possible that the effects on the anterior CNS of XLPA<sub>1</sub>-depletion could be caused by defects in the cortical actin network. To test this, XLPA<sub>1</sub> MO and RLDX were injected at the 8-cell stage into either a dorsal, animal blastomere, to target the CNS, or into a ventral, animal blastomere, to target the epidermis. Embryos were fixed at the mid-neurula stage, and the actin cytoskeleton was stained with Alexa-488 phalloidin. The developing neural plate and epidermis were imaged by confocal microscopy (Fig. 4A).

At the mid-neurula stage, cells throughout both the neural plate and the epidermis had a dense, homogenous cortical actin network (Fig. 4B – upper panel). Occasionally, dividing cells were seen with the less dense cortical actin network characteristic of dividing blastomeres at the blastula stage (Fig. 4B – right, lower panel, arrow). No differences were found in cortical actin assembly, either in the neural folds or epidermis, between controls and XLPA<sub>1</sub>-depleted embryos (Fig. 4B). These data, together with the fact that the neural tube closes normally in XLPA<sub>1</sub>-depleted embryos, shows that the XLPA<sub>1</sub> receptor alone is not necessary to maintain the cortical actin network in cells of either the neural plate
or in the epidermis at the neurula stage. These findings were obtained in three independent experiments.

**XLPA1 is required to inhibit apoptosis and maintain proliferation in the developing anterior central nervous system.**

In vitro, LPA signaling has been shown to activate survival and proliferation pathways and to promote gene expression (Contos et al., 2002; Fukushima et al., 1998; Goetzl et al., 1999; Kingsbury et al., 2003; Weiner and Chun, 1999; Weiner et al., 2001). The loss of anterior CNS structures seen at late tailbud stages could be due to several reasons, including death of the early neural precursors, failure to proliferate, or failure of these cells to differentiate into neurons, instead giving rise to other structures. Analysis of sections from XLPA1 MO-injected embryos at earlier stages revealed fragmenting cells within the anterior CNS that resembled cells undergoing apoptosis (Fig. 2I and data not shown). Therefore, we performed TUNEL staining at various stages.

By the end of neurulation (stage 18), very little apoptosis is still occurring within the normal *Xenopus* embryo (Fig. 5A—left panel, see also (Hensey and Gautier, 1998) and most of this is confined to the anterior CNS. In embryos depleted of XLPA1, there was a significant increase ($p < 0.05$, $n = 20$ per group) in the percentage of cells undergoing apoptosis within the clone of descendants of the injected blastomere in the anterior CNS (Fig. 5A—middle panels, upper arrow marks CNS). If the clone overlapped epidermal cells (Fig. 5A, middle, lower panel, lower arrow) or if the XLPA1 MO was injected into a ventral, animal
blastomere, there was no significant increase in apoptosis within the epidermis (data not shown). At stage 25 after the onset of eye induction, apoptosis levels were still significantly high in XLPA\textsubscript{1}-depleted cells within the anterior CNS compared to controls. Apoptotic cells were occasionally seen in the spinal cord, although these embryos could respond to touch and could swim normally, suggesting that the degree of apoptosis in the spinal cord was not sufficient to abrogate function. At stage 28-9, apoptosis was still increased on the injected side within the forebrain compared to the uninjected side (data not shown). These data show that the absence of forebrain structures in XLPA\textsubscript{1} MO-injected embryos was due to a loss of neural cells through apoptosis. These effects could be rescued by reintroduction of XLPA\textsubscript{1} mRNA with a significant decrease in the amount of TUNEL staining (Fig. 5A, D). Control embryos at stage 18 were found to have a mean of 19.1 TUNEL-positive nuclei (Measured as the number of TUNEL-positive nuclei on one side of the anterior CNS). This increased to a mean of 78.1 positive nuclei on the injected side in the XLPA\textsubscript{1} MO-depleted embryos, and was rescued to 34.3 positive nuclei in the rescue group (* indicates p < 0.05). This rescue correlated with a restoration of CNS structures. This finding was obtained in three independent experiments.

During normal development, cells in the anterior CNS proliferate for a longer period than more posterior cells before exiting the cell cycle and differentiating (Eagleson et al., 1995; Hartenstein, 1989; Hartenstein, 1993; Papalopulu and Kintner, 1996). To test if LPA signaling plays a role in maintaining cell proliferation in the anterior CNS, we examined the
phosphorylation of histone H3 (HP3) on Ser10. This is an established marker for actively cycling cells, from late G2 of the cell cycle until telophase of mitosis (Hama et al., 2004; Hendzel et al., 1997; Paulson and Taylor, 1982). At stage 22, high levels of proliferation were found throughout the embryo (Fig. 5B, left panel). In XLPA₁-depleted embryos, there was no detectable difference in rates of cell proliferation outside the CNS. However, within the lateral, anterior nervous system (Fig. 5B, arrow), there was a 14% decrease in cell proliferation rate in XLPA₁ MO-injected embryos compared to controls (n = 20 embryos, p < 0.05; Fig. 5B, measured as the number of HP3-positive cells out of the total number of cells in a 0.15 mm² area in the anterior CNS). After sectioning the embryos at the level of the developing forebrain, almost no proliferating cells were found within the clone on the injected side (Fig.5C). There was an average of 21 HP3-positive cells per section in the anterior CNS of XLPA₁-depleted embryos. Of these cells, 84% were located outside of the clone on average while a mean of 16% were descendants of XLPA₁ MO-injected cells (Fig. 5E, * indicates p < 0.05). This result was obtained in three independent experiments. These data suggest that XLPA₁ is necessary for both maintaining a proliferating population of cells and inhibiting apoptosis within the anterior CNS.

**Expression of genes in the anterior CNS is decreased in XLPA₁ MO-injected embryos, while patterning is unaffected posteriorly.**

Early in development, attenuation of BMP signaling in the dorsal ectoderm leads to induction of this tissue to adopt a neural fate. Neural induction leads to
expression of the Group B1 Sox transcription factors, Sox2 and Sox3 (Kuroda et al., 2004). At early neural stages, Sox2 is expressed throughout the entire CNS. In XLPA1 MO-injected embryos, there was no change in Sox2 expression during early stages of neurulation (data not shown). In control embryos, at stage 22 when eye formation has begun, Sox2 was still found ubiquitously within the CNS and expressed in the presumptive lens ectoderm (Fig. 6A, left panel). In embryos injected with XLPA1 MO, there was a loss of Sox2 expression specifically in the anterolateral CNS, including the lens placode and optic vesicle (Fig. 6A arrow in center panel, n = 38, 87% affected). The majority of this domain was absent in injected embryos, while the medial, anterior CNS maintained expression of Sox2 at this stage. The expression of Sox2 was somewhat reduced and mislocalized in hindbrain regions, but unaffected in the spinal cord (Fig. 6A, right panel). Both Pax6 and Otx2 are expressed within the developing anterior CNS at the time of eye formation (Acampora et al., 1995; Andreazzoli et al., 1997; Hirsch and Harris, 1997; Pannese et al., 1995). In XLPA1 MO-injected embryos, the lateral domains of both Pax6 (Fig. 6C,D, n = 30, 88%) and Otx2 (Fig. 6E, n = 30, 87%) were severely reduced, but the medial expression pattern was unaffected, similar to that of Sox2. Also, at stage 22 Pax6 was expressed in specific rhombomeres within the hindbrain (Fig. 6D, black arrowhead) and this pattern was reduced in injected embryos. Rx, an early marker of the eye field (Mathers et al., 1997), was expressed in XLPA1-depleted embryos in the eye field, but it was reduced and more medial (Fig. 6F, n = 24, 79% affected). Therefore, markers of the anterior,
lateral CNS were decreased or absent in XLPA<sub>1</sub> MO-injected embryos, but the posterior CNS expression was unaffected.

To test whether XLPA<sub>1</sub> is required for the patterning of mid- or hind-brain structures, we analyzed the expression of two markers. *En2* is expressed at the midbrain-hindbrain junction and *krox20* is expressed in rhombomeres 3 and 5 (Hemmati-Brivanlou et al., 1991; Macrae et al., 1996). Expression of both *en2* (Fig. 6B, n = 8, 100%) and *krox20* (data not shown) were unaffected or slightly reduced in XLPA<sub>1</sub>-depleted embryos suggesting that XLPA<sub>1</sub> is not required for the expression of these mid- and hindbrain markers. To test whether XLPA<sub>1</sub> is required for dorsal-ventral patterning, expression of two genes was examined. *Cpl-1* is a marker of the dorsal neural tube and *nkx2.1* is a marker of ventral forebrain (Knecht et al., 1995; Small et al., 2000). In embryos lacking XLPA<sub>1</sub>, *cpl-1* and *nkx2.1* were still expressed, but reduced at stage 22 on the injected side (Fig. 6G, H, n = 50, 84%).

Finally, we examined the expression of *Neural*-tubulin to determine if primary neurogenesis was also disrupted. When neurons first form during neurulation, three primary neuron tracts can be identified, corresponding to a lateral domain of sensory neurons, a more medial domain of interneurons, and a midline domain of motor neurons, that run from anterior to posterior (Fig. 6I and (Chitnis et al., 1995). This pattern was disrupted in XLPA<sub>1</sub> depleted embryos, starting at the early neurula stage (stage 14). At this stage, the interneuron domain was most affected and both the midline and lateral neuron domains were reduced (Fig. 6I). By stage 22, the neural tube had formed in control embryos.
and the three earlier neuronal tracts now appeared as a single, sagittal stripe running posteriorly along the dorsal midline (Fig. 6J, left panel). In the anterior CNS, there were two positive lateral domains in the optic vesicle and a more medial area that corresponds to the anterior-most expression of the motor neuron column (white arrowhead). In the XLPA₁ MO-injected embryos, mature neurons formed within the optic vesicle, but were displaced laterally and shaped abnormally (Fig. 6J right panel, n = 23, 91%). Staining in the midbrain and forebrain was reduced, but more posterior staining was unaffected (Fig. 6J right panel). These data suggest that some neuronal differentiation occurs, but that there are fewer neurons in the forebrain, and the tracts had either become mis- localized, or the cells contributing to these tracts had died.

Discussion

In previous work, we showed that blockade of XLPA₁ and XLPA₂ synthesis from both maternal and zygotic mRNAs caused a decrease in the density of the cortical actin cytoskeleton in cells of the blastula, which resembled blastomeres that had rounded up to divide. When these embryos were allowed to develop to later stages, numerous defects were seen, which appeared to involve many emerging organ systems and developmental processes (Tao et al., 2005). In this work, we show that these post-gastrulation defects can be dissected using morpholinos to block zygotic, rather than maternal receptor synthesis, and making use of the well established *Xenopus* fate map to target individual receptors in restricted cell lineages. Targeting of the XLPA₁ MO to descendants
of a single dorsal, animal blastomere at the 8-cell stage caused dramatic defects in the anterior CNS.

It is interesting that targeting of the morpholino does not lead to defects in gastrulation, formation of the neural plate, or in neural induction (based on the expression of early neurogenic genes). However, shortly after the specification of the neural plate, as judged by the expression of Sox2 and other CNS markers, XLPA₁ becomes necessary for the survival of neurogenic cells forming the forebrain and optic cup. These degenerate by apoptosis, until, in the most severe cases, these structures are completely absent by the swimming tadpole stage. Quantitation of cells in the cell cycle shows that the cell division rate is also reduced in the surviving cells. This suggests that LPA signaling through XLPA₁ is an absolute requirement for cell survival and proliferation in this restricted region of the CNS. This is the first demonstration of an essential role for LPA receptors during early organogenesis.

Descendants of the dorsal, animal blastomere at the 8-cell stage also contribute to head epidermis and mesenchyme, neural crest, notochord, and lens tissue (Moody and Kline, 1990). Although RLDX-positive (and thus MO-injected) cells were detected in these tissues, they were histologically normal. This could be due to the fact that other blastomeres also make substantial contributions to these tissues and may compensate for the loss of XLPA₁, or that XLPA₁ is not required for cell survival in these tissues. To test this, we targeted XLPA₁ to a single ventral, animal blastomere, which gives major contributions to the epidermis on one side of the body, neural crest, cranial ganglia, and lens tissue.
No increase in apoptosis was found in the targeted epidermis. The tissue differences seen in the XLPA\textsubscript{1} depletion are not due to differential expression, since the transcript was expressed in all early germ layer derivatives based on real-time RTPCR analysis of tissue explants. The differential effects must therefore be due to activation of different pathways downstream of XLPA\textsubscript{1} in a tissue-specific manner or differential roles of other lipid receptors in these tissues.

The defects in the anterior CNS are not due to a reduction in cortical actin density or changes in actin dynamics. Depletion of both maternal and early zygotic mRNAs of both XLPA\textsubscript{1} and XLPA\textsubscript{2} showed that LPA signaling is required to maintain a dense actin cytoskeleton during the blastula stage (Tao et al., 2005). However, analysis of cortical actin during the neurula stage in embryos depleted of XLPA\textsubscript{1} showed no defects in actin assembly in the neural plate. This suggests that either other receptors may be required in addition to XLPA\textsubscript{1} (XLPA\textsubscript{2} is also expressed in the neural plate) or that actin assembly in the neural plate is controlled by other signals instead of, or in addition to, LPA. The lack of a cortical actin defect is consistent with the facts that neural tube and convergent extension movements occur normally, and the posterior CNS is unaffected.

It is intriguing that the defects seen were specific to the anterior CNS compared to the posterior CNS. Neural tissue begins responding differently along the anterior-posterior axis to different signals after induction. Several signaling pathways intersect to confer an anterior identity, resulting in the expression of anterior specific factors, including FoxG1 (Bf1), Otx2, Six3, and Rx (Acampora et
al., 1995; Andreazzoli et al., 1999; Andreazzoli et al., 1997; Bourguignon et al.,
1998; Lagutin et al., 2003; Lynch et al., 1997; Mariani and Harland, 1998;
Mathers et al., 1997). Additionally, cells in the anterior CNS are held in a state of
prolonged proliferation and delayed neuronal differentiation compared to the
posterior CNS and this is crucial for proper anterior formation (Eagleson et al.,
1995; Hartenstein, 1989; Hartenstein, 1993; Papalopulu and Kintner, 1996;
Wilson and Houart, 2004). Conversely, posterior cells respond to retinoic acid
and other signals, which induces exit from the cell cycle and terminal
differentiation earlier than the anterior CNS. Because of these inherent
differences in signaling, it is possible that survival signals regulate these tissues
differently. In the anterior CNS, LPA may be the primary survival signal, while in
the posterior other signals may be required. LPA may also be a survival signal
that is downstream of the signals that cause continued proliferation of the
anterior CNS, and this does not become established as a survival mechanism in
the posterior CNS.

Loss of XLPA\textsubscript{1} in the CNS leads to apoptosis that begins during late
neurulation and continues throughout the tailbud stages. Based on histology,
nuclei become pyknotic and fragment within the CNS as neurulation is completed
in the neural tube and optic vesicle. The substantial increases in apoptosis are
seen only in the anterior CNS and not other tissues. In the LPA\textsubscript{1} mouse
knockout, increased levels of apoptosis were detected in sciatic nerve Schwann
cells, but these increased levels did not lead to defects in motor reflexes or nerve
dysfunction (Contos et al., 2000). It is possible that in the \textit{Xenopus} posterior
CNS, and these Schwann cells, that other survival factors may compensate for the loss of XLPA\(_1\). It is not known which pathways prevent cell death downstream of XLPA\(_1\) in the anterior CNS. It has been shown in wild-type MEFs that Akt and JNK are phosphorylated in response to LPA and that this response is lost in MEFs that are null for LPA\(_1\) and LPA\(_2\) (Contos et al., 2002). Analysis of a T-lymphoblastoma line that expresses both LPA receptors shows treatment with LPA causes decreased apoptosis via a reduction in the pro-apoptotic protein bax without changes in other pro-apoptotic or anti-apoptotic proteins (Goetzl et al., 1999). It is possible that XLPA\(_1\) works through similar pathways to control cell survival in the CNS.

It is clear from these results that requirements for XLPA\(_1\) in the early CNS are selective. There is no effect on either neural induction or on formation of the neural tube. In addition, molecular markers of anterior CNS, such as Otx2, and eye differentiation continue to be expressed, even during the massive waves of apoptosis caused by XLPA\(_1\) depletion. Many molecular markers are expressed ectopically or at decreased levels, suggesting that LPA signaling plays a primary role in cell survival of progenitor cells in the early CNS, rather than in their differentiation. This can only be confirmed by experiments in which apoptosis is blocked in these embryos.

In conclusion, these experiments demonstrate an early role for signaling through XLPA\(_1\) as an essential survival signal in the anterior CNS in *Xenopus* development. By targeted depletion and clonal analysis, we have been able to elucidate roles for this receptor within the CNS, not previously realized in other
vertebrate models because of redundancy of signaling. In the future, it will be interesting to identify the pathways downstream of XLPA1 in the anterior CNS and the roles of other lipid signals, and their receptors, during post-gastrula lineage divergence in the *Xenopus* embryo.

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**References**


**Figure legends**

**Fig. 1.** Localization of *Xenopus* LPA receptor transcripts during early post-gastrula development. (A) Quantitative real-time RTPCR in cultured tissue explants. Explants were cut at stage 9 from 10 embryos and cultured until stage 19. RNA was isolated and cDNA synthesized for real-time RTPCR (Left panel). Both XLPA\(_1\) and XLPA\(_2\) mRNA are expressed in all of the explants at similar levels (middle panel). *Epidermal keratin* mRNA is enriched in the animal cap, *Xbra* mRNA in the equator, and *endodermin* mRNA in the base (Right panel). B-E. In situ hybridization for XLPA\(_1\) and XLPA\(_2\). (B) XLPA\(_1\) mRNA is expressed ubiquitously in the developing central nervous system throughout neurulation. Early neurula (stage 14, anterior view – left) and late neural (stage 18: anterior view – middle and dorsal view – right). (C) XLPA\(_2\) mRNA is also expressed in the CNS during early neurula (left) and late neurula (right) stages. (D) XLPA\(_1\) mRNA sense control at stage 14. (E) At tailbud stages, XLPA\(_1\) (upper panel) mRNA is enriched in the branchial arches (b), CNS, otic vesicle (o), and in a segmental pattern along the dorsal region. XLPA\(_2\) (lower panel) is expressed in the eye and throughout the CNS. (F) Sense control for XLPA\(_2\) mRNA at stage 28. (G) Transverse section at the level of the thoracic spinal cord from an XLPA\(_1\) in situ in (E) showing staining in the somites. Scale bar in B-F: 140 \(\mu\)m.

**Fig. 2.** Loss of XLPA\(_1\) leads to defects in the anterior CNS. (A) Bright-field image of a control embryo at stage 15 (mid-neurula) and (B) corresponding RLDX injection in the CNS. (C) Control embryo at the tadpole stage (stage 38) showing
normal eye morphology. (D) An XLPA₁ MO injected embryo with reduced eye. RLDX (red) was coinjected with the MO as a lineage tracer. (E) Overexpression of a MO-resistant mRNA for XLPA₁ that lacks the endogenous 5' UTR does not cause CNS defects or loss of eyes. mRNA encoding GFP was coinjected as a lineage tracer. (F) Rescue of the XLPA₁ MO phenotype showing restored eye structures and the location of the injection. (G) The same embryo in F showing the location of the subsequent injection of the rescuing mRNA. Scale bars in A-G: 250 µm. (H) XLPA₂ MO injected embryos do not have defects in CNS formation. (I) Histology of the XLPA₁ MO phenotype in transverse sections taken at the level of the forebrain. Arrow indicates injected side. Degeneration is seen in the neural tube and eye in XLPA₁-MO injected embryos (second panel) and is rescued (fourth panel). (K) Transverse sections were cut from a tailbud stage embryo at the indicated regions and shown in (J). Defects are seen in the neural tube at the level of the hindbrain (a), but are not apparent in the posterior CNS (spinal cord - b). (L) Quantitation of the phenotypes in C – G. Embryos were divided into normal, mild (50 – 99% of normal eye diameter present), and severe (< 50% of normal eye diameter present) groups. There is a dose-dependent increase in the mild and severe groups with MO injection. There is a significant (p < 0.05) rescue in the 30 ng MO dose after subsequent injection of a rescuing mRNA.

**Fig. 3.** The XLPA₁ phenotype is not rescued by XLPA₂ mRNA. Quantitation of the XLPA₂ rescue. Embryos were divided into normal, mild (50 – 99% of normal
eye diameter present), and severe (< 50% of normal eye diameter present) groups. There is no effect of the XLPA2 mRNA alone on either forebrain or eye development. There is a significant (p < 0.05) increase in the severe phenotype in both the XLPA1–depleted embryos (55%) and the XLPA2 mRNA rescue (40%).

**Fig. 4.** Depletion of XLPA1 in the neural plate or in the epidermis does not cause decreases in the density of cortical actin. (A) A mid-neurula stage embryo showing the two views examined. View 1 is of the anterior neural plate and view 2 is of the ventral epidermis. (B) Actin staining of a neurula from the two views in A. Upper panels show the actin cytoskeleton in control embryos (left) and embryos that were depleted of XLPA1 in either the CNS (middle) or epidermis (right). Lower panels show the same conditions, but a ventral view. Scale bar: 10 µm.

**Fig. 5.** CNS defects in XLPA1-depleted embryos are due to specific increases in apoptosis in the CNS and decreased cell proliferation. (A) TUNEL staining in XLPA1 MO-injected embryos. Images are an anterior view (See left, lower panel – arrow indicates anterior CNS). Left, upper panel shows representative TUNEL staining in the anterior CNS of a stage 18 embryo (just after neurulation). Middle panels demonstrate increased levels of TUNEL staining on the injected side versus the control side. White is TUNEL (Upper, middle panel) and red indicates location of XLPA1 MO (Bottom, middle panel). Upper arrow indicates CNS and lower arrow denotes where some of the injection was located in the ventral
epidermis (ep). Notice there are no increases in apoptosis in the epidermis where the MO was injected. Right panels show a rescue embryo with decreased levels of apoptosis on the injected side. (B) HP3 staining at stage 20. In the control (left panel), there are many HP3 positive nuclei detected (blue, anterior view). Center and right panels demonstrate decreased levels of HP3 staining on the injected side of an XLPA1 MO-depleted embryo that are significant within the anterolateral domain of the CNS (arrow). (C) Immunofluorescence for HP3 on transverse tissue sections from the forebrain region of stage 22 embryos. Upper left panel shows HP3 positive nuclei (Blue), upper right panel shows the location of the XLPA1 MO, lower left panel is a Sytox green stain for nuclei, and the lower right panel is the merge. HP3 positive cells are mostly detected outside of the XLPA1 MO clone. Arrowhead denotes the forming optic vesicle (ov). (D) Quantitation of the number of apoptotic nuclei in XLPA1-depleted embryos. In control embryos, there is an average of 19 TUNEL positive nuclei on one side of the embryo at stage 20. This number increases to 78 positive nuclei in MO injected embryos and can be rescued to an average of 34 positive nuclei in the rescue group (* p < 0.5). (E) In each section, there is a mean of 21 positive HP3 cells. Of the positive cells, a mean of 84% cells are detected outside of the clone (* p < 0.05) while only 16% are in the clone. Many sections contain no proliferating cells within the clone. Scale bars in A, B: 200 µm and 100 µm in C.

**Fig. 6.** Expression of anterior neural markers is reduced in XLPA1-depleted embryos. A,B,D,E,F,G,H,J are at stage 22 of development when eye induction occurs, while C and I are at stage 14 of early neurulation. White arrows indicate
injected side. Embryos in F-H were cleared in Murray’s clear. (A) Sox2 is expressed in all neural tissue at stage 22. Control (left), and XLPA1 MO injected embryo (middle panel, anterior – right panel, dorsal) showing a loss of the anterolateral domain of expression. (B) Expression of En2 mRNA at the midbrain-hindbrain junction is unaffected in XLPA1 MO depleted embryos. Both the early (C) and late (D) expression patterns of Pax6 are disrupted on the injected side. Arrowhead in D denotes expression in rhombomeres. (E) The anterior marker Otx2 is absent in the from the developing eye region, but still present in the midline of the anterior CNS. (F) Rx is present in XLPA1 MO injected embryo, but reduced on the injected side. (G) A marker of the dorsal CNS, Xcpl1 or lipocalin, is reduced on the injected side and displaced laterally indicating that dorsal-ventral patterning has occurred, but the tissue is misplaced. (H) The ventral CNS marker, Nkx2.1, is also reduced on the XLPA1 MO-injected side at stage 22. (I) N-tubulin, a marker of primary neurons, is expressed in three tracts that run anterior to posterior (Left panel, control). From lateral to medial these tracts represent sensory neurons (s), interneurons (i), and motor neurons (m). In embryos depleted of XLPA1, the motor and sensory tracts are reduced while the interneuron tract is abolished. (J) At later stages, N-tubulin expression is abnormally placed in the eye field, while expression in the developing neural tube is reduced or absent in XLPA1 MO-injected embryos. More posterior domains of expression are unaffected. Scale bar in A-J: 250 µm.
**FIGURE 1**
FIGURE 2
FIGURE 5

A
Control  XLPA1 MO  Rescue

B  Control  XLPA1 MO  XLPA1 MO

C

D

E

% of apoptotic nuclei

% HPJ positive cells in clone

% HPJ positive cells outside clone

Control  XLPA1 MO  Rescue

* *
CHAPTER IV

Unpublished data.
Introduction

In addition to the experiments presented in the previous chapters, further work has been performed to clarify mechanisms and support the hypotheses of a role for LPA signaling in controlling actin levels and later as a survival signal in CNS development. In mammalian models, S1P receptors are thought to compensate for the loss of LPA receptors. Here we present further data characterizing the expression of a *Xenopus* S1P receptor and analyzing the ability of sphingosine signaling to regulate actin dynamics.

In the first manuscript, LPA signaling was modulated by adding ligand, overexpressing receptors, and performing loss-of-function analysis with the receptor. *In vivo*, a class of enzymes, lipid phosphate phosphohydrolases (LPPs), exist that degrade extracellular LPA and have the potential to regulate the level of LPA signaling. Here we begin to characterize the ability of these enzymes to control LPA levels, and thus LPA signaling.

Finally, the second manuscript focused mainly on depletion of XLPA\textsubscript{1} within the CNS, but since the receptor is expressed in all developing germ layers it may have other roles in different tissues. We show additional phenotypes when the XLPA\textsubscript{1} MO is targeted to ventral, animal cells.

A single sphingosine-1-phosphate receptor is expressed beginning at gastrulation in *Xenopus laevis*

It is possible that other lipid receptors are expressed in the developing embryo and these may compensate for loss of the LPA receptors or influence
similar developmental processes as XLPA\textsubscript{1} and XLPA\textsubscript{2}. To determine if other LPA or S1P receptors are expressed in \textit{Xenopus}, we performed a BLAST search of the mammalian isoforms against the \textit{Xenopus laevis} and \textit{Xenopus tropicalis} EST databases. From this search, we identified a single S1P receptor, but no other LPA receptors. This S1P receptor had the highest homology to S1P\textsubscript{1} and so we named it XS1P\textsubscript{1}. This receptor contains the three conserved amino acids that are characteristic of an S1P receptor and it clusters in the S1P subfamily of GPCR (Fujiwara et al., 2005; Sardar et al., 2002; Tigyi et al., 2000).

To determine when in development XS1P\textsubscript{1} mRNA is expressed, we performed quantitative real-time RTPCR on the explant series used for the LPA receptors (See Chapter III, page 82, 85). XS1P\textsubscript{1} mRNA was not expressed maternally, rather expression began at the commencement of gastrulation at low levels (Fig. 1). During the neurula stages, expression of XS1P\textsubscript{1} mRNA increased and there was a second increase in expression again at the onset of organogenesis (Stage 25). We wanted to determine if XS1P\textsubscript{1} mRNA was expressed in a tissue-specific manner or if it was ubiquitous similar to the LPA receptors. RTPCR was performed on tissue explants from caps (ectoderm), equators (mesoderm), and bases (endoderm) that were incubated until stage 19. XS1P\textsubscript{1} mRNA was expressed in all of the explants and expression was highest in animal caps and equators (Fig. 2), but lowest in the bases. Therefore, in addition to the two LPA receptors, a single S1P receptor is expressed beginning at gastrulation in all developing germ layers in \textit{Xenopus}. 
Figure 1. XS1P1 mRNA is expressed zygotically throughout tadpole stages. Quantitative real-time RTPCR.

Figure 2. XS1P1 mRNA is expressed in all germ layers of the developing embryo. Quantitative real-time RTPCR in cultured tissue explants. XS1P1 mRNA is expressed highest in the caps and equators.

Figure 3. S1P signaling does not affect actin dynamics at the blastula stage. A. Control animal cap stained for F-actin. B. An animal cap that healed for 10 minutes in 5 uM S1P does not show changes in cortical actin. C. A control cap and (D) an animal cap depleted of XS1P1 have similar actin cytoskeletons and wounding responses. Scale bar – 20 μm.
**S1P signaling does not affect the density of the actin cytoskeleton.**

Though both XLPA receptors were shown to control the density of cortical actin at the blastula stage, other mechanisms may regulate actin dynamics. Once gastrulation begins, it is possible that XS1P₁ may also influence actin. To test if S1P was able to change the actin network, isolated animal caps from late blastulae were incubated in either 5 or 25 µM S1P for 10 minutes, caps were fixed, stained with Alexa 488-phalloidin, and imaged (See Chapter II for methods). The density of the actin network is similar in control caps that were allowed to heal for 10 minutes compared to caps that were allowed to heal in the presence of either dose of S1P (Fig. 3A compare left to right). Both of these caps healed at the same rate and cells had the same morphology in each group. So, S1P is not able to influence actin dynamics during the stages when S1P receptors are expressed.

Additionally, depletion of XS1P₁ may affect the actin cytoskeleton. To determine if XS1P₁ is necessary for actin dynamics at this stage, 10 ng of an XS1P₁ MO, targeting the ATG start site of the mRNA, was injected into both cells at the 2-cell stage, embryos were incubated until the onset of gastrulation, and caps were excised and stained as before. Depletion of XS1P₁ did not cause actin defects compared to controls (Fig. 3B). Actin density, cell morphology, and process formation were similar in both control animal caps and XS1P₁-depleted caps. Therefore, XS1P₁ does not influence actin density at the beginning of gastrulation and the actin phenotype is specific for LPA receptors.
The density of the actin network may also be affected *in vivo* by altering the levels of ligand.

Since the XLPA receptors are expressed ubiquitously, regulation of LPA signaling is most likely controlled either upstream or downstream of the receptor. It has been proposed that possible ways to control lipid signaling are via the production and termination of the ligand. LPPs are a class of six-transmembrane domain containing proteins with the active site facing the extracellular matrix and may dephosphorylate, and thus inactivate, LPA. LPPs dephosphorylate several lipids including phosphatidic acid (PA), LPA, S1P, and ceramide-1-phosphate (C1P) (Le Stunff et al., 2002; Pyne et al., 2005). We showed indirectly that LPA is present at the blastula stage. To determine if lowering LPA levels was also able to influence the actin cytoskeleton, we injected mRNA encoding hemeagglutinin (HA) tagged LPP enzymes (1-3) into both blastomeres at the 2-cell stage, isolated caps from injected embryos at the blastula stage, and stained for cortical actin.

If LPPs control the level of LPA in the early *Xenopus* embryo, then increasing the amount of XLPP may decrease the level of extracellular lipids available to the receptors which would reduce actin density. Overexpression of any of the three XLPP isoforms resulted in a reduction in the intensity of cortical actin staining that is similar to loss of XLPA receptors (Fig. 4A). The most profound reductions in actin were seen in caps overexpressing XLPP3. In all of the caps, an actin purse-string was present, supporting the fact that LPA
Figure 4. Overexpression of *Xenopus* LPPs decreases the amount of cortical actin. A. Low power view of caps overexpressing LPPs. There is a decrease in actin in all of the injected caps compared to control. Scale bar – 10 μm. B. High power view of a control cap (left) and an XLPP3-HA injected cap (right). There is a significant decrease in actin staining in all blastomeres. Scale bar – 5 μm. C. Quantitation of the intensity of phalloidin staining. There is a significant decrease in phalloidin staining in all caps overexpressing XLPPs (* p < 0.05).
signaling is not necessary to control the formation of an actin-purse string. These caps were able to heal at the same rate as control caps. At higher power, the cells in the cap overexpressing XLPP3-HA had reduced actin density and these cells had a more rounded morphology (Fig. 4B). Cell-cell contacts were present in the cap as no cells were dissociating from the cap. Additionally, cells extended fewer processes compared to control caps. The reduction in actin staining intensity was reduced significantly (Fig. 4C p < 0.05) and the highest decrease was in caps expressing XLPP3-HA mRNA. Therefore, reduction of the ligand LPA by overexpressing the degradation enzymes leads to a decrease in the density of the actin cytoskeleton that is similar to caps that are depleted of XLPA receptors.

**ROCK, a downstream effector of Rho, is necessary to mediate some of the overexpression effects of XLPA receptors**

Both the small Rho GTPases Rho and Rac are required for the overexpression effects of XLPA<sub>2</sub> (Lloyd et al., 2005). Based on work in cell culture, Rho-associated cellular kinase (ROCK), is the downstream effector of Rho that regulates actin-myosin dynamics by phosphorylating myosin light chain kinase and myosin phosphatase to activate and inactivate them, respectively (Amano et al., 2000). This leads to efficient cross-bridging between actin and myosin so that contraction may occur (Darenfed and Mandato, 2005). A small molecule inhibitor is available that inhibits ROCK, Y27632 (Fig. 5A), and it has been used in many studies to demonstrate a role for this protein in several
pathways (Uehata et al., 1997). Recently, a second small molecule inhibitor was synthesized that is more specific for ROCK and can be used in smaller doses to achieve inhibition, H1152 (Ikenoya et al., 2002; Sasaki et al., 2002).

To determine if ROCK is required downstream of an LPA signal, the vitelline membrane was removed from embryos during early blastula stage, embryos were transferred into either a 1 or 10 µM solution of H1152, and incubated for three hours. Animal caps were cut from these embryos and stained as before for F-actin (Chapter II). Animal caps that were treated with H1152 had impaired wounding responses compared to control caps. These animal caps did not form a purse-string and did not round up after excision (Fig. 5B). The bases from these embryos did not heal and embryos were less rigid than controls. The H1152-treated caps had decreased amounts of F-actin in all cells and the cells had a tight interlocking cell morphology that was similar to animal caps that were overexpressing RhoA-N19 (Lloyd et al., 2005). These data suggest that ROCK is necessary for purse-string formation, increased density in the actin cytoskeleton, and for wound-healing.

Animal caps overexpressing XLPA2 mRNA and treated with H1152 showed some of the overexpression effects, but not all. These caps had cells with very distinct, rigid borders and there was an increase in actin levels throughout the cap (Fig. 5B). The cells did not extend any processes and no purse-string formed in the exterior-cells. The increase in actin was probably mediated through a Rac-dependent pathway, since this protein still functions in the presence of H1152. So, the LPA signal was able to increase actin levels and
affect cell rounding when ROCK was blocked, but the LPA signal was not able to restore the purse-string or restore wound-healing.

**Targeting the XLPA₁ MO to a ventral, animal cell disrupts melanocytes at later stages of development**
When the XLPA₁ MO was targeted to a ventral, animal cell at the 8-cell stage, no defects were apparent at gross or histological levels in the epidermis (Fig. 6A-C). The epidermis was not breeched or degraded and there were no signs of pyknotic nuclei in the epidermis, based on DAPI staining from neurulation to stage 45 (tadpole). The only evident defect in these embryos was apparent when pigment started to appear. Control embryos had pigmented cells on the dorsal aspect of the head, along the dorsal axis, and over the dorsal aspect of the gut (Fig. 6A,B).

Figure 6. Injection of XLPA₁ MO into a ventral, animal cell causes melanocyte defects. A. Control embryo injected with RLDX at the tadpole stage showing distribution of the tracer throughout the epidermis. B. (upper) Injected side showing decreased melanocytes over the dorsal hindgut (arrow) and dorsal axis. (lower) Uninjected side of the same embryo for comparison. C. Tissue section from an injected embryo showing distribution of the XLPA₁ MO (Red) and a nuclear stain (DAPI – blue). No defects are present in either the eye or epidermis.
In embryos injected with XLPA\textsubscript{1} MO on the ventral side, there was a lack of pigment in all of these areas on the injected side, but pigment was present on the uninjected side. In these embryos, the MO was incorporated into head mesoderm, based on the location of RLDX (Fig. 6B). Even though MO was present in the mesoderm surrounding the eye, the eye did form normally and was patterned correctly. Based on these data, a second role for signaling through XLPA\textsubscript{1} may be in neural crest cells which give rise to the pigmented melanocytes.

References


The work presented here shows the following:

1. Intercellular signaling is required to maintain a dense cortical actin network in cells of the *Xenopus* blastulae.

2. Increased levels of LPA signaling, either by adding LPA ligand or by overexpressing LPA receptors, leads to increased actin density, increased purse-string density, and expedites wound-healing.

3. Depletion of LPA receptors at the blastula stage results in a decrease in the actin density, to a level similar to either dissociated or dividing cells.

4. Addition of LPA to dissociated cells is able to restore the actin density to normal levels.

5. The small Rho GTPases Rho and Rac (but not cdc42) and the Rho effector ROCK are necessary for the cytoskeletal overexpression effects of XLPA receptors.

6. One *Xenopus* LPA receptor is expressed maternally and zygotically while a second LPA receptor and a single S1P receptor are expressed in all developing germ layers of the developing embryo after the mid-blastula transition.

7. XLPA₁ is required for cell proliferation and survival, in the anterior CNS, but not in all tissues of the embryo.

8. XLPA₁ is not required for maintaining the density of the actin cytoskeleton during neurula stages.

9. Depletion of XLPA₁ in the CNS does not affect induction of neural markers, but these are all abnormal or absent during late neural stages.
Intercellular signaling is required to maintain the uniform density of cortical actin in interphase cells of the *Xenopus* blastula

Based on the cell dissociation and reaggregation experiments, an intercellular signal in the form of secreted ligands, cell-cell contact, or possibly both is required to maintain a dense cortical actin cytoskeleton. There is evidence to support both of these mechanisms and it is possible that together these processes are able to coordinate the formation of an actin cytoskeleton that will enable the cells to act as a uniform tissue and survive forces present during morphogenesis.

One possible model for how these two processes could be integrated requires that cell-cell contacts are needed to initiate the change from a sparse to dense actin network (Fig. 1A). Once cell-cell contacts are formed between two cells, an intracellular signal is sent from the adhesion complex to each cell (#1) that would then signal for lipid production and/or release (#2). LPA would be released into the extracellular environment and signal either in a paracrine or autocrine fashion (#3). Thus, LPA would function as a second signal (#4) to cause an increase in the actin density (#5) that is suitable for cells in a network to perform complex tissue movements and to resist shear forces and stresses.

The data presented here give support for such a model, but further experiments are needed to rule out other possibilities. First, when LPA receptors are depleted at the blastula stage, cell-cell contacts must be still present even though cells in the animal cap adopt a coarse network as if they are dissociated
Figure 1. Models for how LPA signaling may regulate actin dynamics.
A. When cell contacts are initiated (1), a signal is sent to the cell (2) to release or synthesize LPA. LPA signals in an autocrine/ or paracrine (3) fashion to the actin cytoskeleton (4). The result is an increase in actin density (5). B. LPA signaling may directly influence cell contacts. LPA signaling would result in the formation of cell contacts and these contacts would orchestrate the formation of a dense actin network.

Figure 2. Several lipid signals may control actin dynamics. When cell contacts are initiated, several lipids may be released and these lipids may control individual aspects of actin dynamics such as increasing density, polymerization, and other functions.
or dividing cells. If contacts were absent, then cells in the embryo would dissociate at this stage, but this is not the case. It is not clear if LPA depletion leads to a change from one type of cell contact to another or if proteins involved in adhesion are up- or down-regulated. Second, the morphology of blastomeres with reduced LPA signaling resembles dissociated or dividing cells. If LPA is a second signal, then it should still be able to upregulate actin regardless of whether cell-cell contacts are present. When LPA is added to an intact animal cap or to isolated blastomeres, there is an upregulation in the amount of cortical actin. It will be interesting to determine if LPA may still exert its effects in animal caps depleted of cell adhesion molecules, such as cadherins. These data suggest that LPA may act as a second signal to upregulate the level of cortical actin and thus as a mediator of cell-cell contact.

It is possible that LPA signaling may function upstream of cell-cell contacts. In this model, receipt of an LPA signaling may influence cell-cell contacts, either by upregulating cell adhesion complexes or transporting these complexes to the cell membrane (Fig. 1B). Once the adhesion complexes formed between cells, these complexes would serve as a foci for actin assembly and lead to the development of a dense actin network. A third possibility is that LPA signaling is independent of cell contact and that other signals regulate LPA synthesis, release, and signaling.

Further experiments may be carried out to determine the role of cell-cell contacts in this model. One possibility is that adhesion complexes, such as the cadherin complex, may be able to signal through the LPA pathway by mobilizing
pathway components, increasing LPA production, synthesizing LPA receptors, or via a combination of these mechanisms. When cell-cell contacts are formed, it is possible that several lipids are formed to influence and execute different aspects of actin dynamics. Recently, a second orphan GPCR, XFlop, has been described that is necessary to regulate the level of cortical actin in the cell (Tao et al., 2005). Formation of cell contacts may also produce the ligand for Xflop to upregulate enough actin for a dense network, as well as other regulators (Fig. 2).

This proposed mechanism may be instrumental in preparing cells of the blastula for tissue movements. Tissues at the blastula stage will begin to undergo complex movements including epibole, ingression, involution, and convergent extension, and many forces will be applied to these tissues (Keller, 2005; Komazaki, 1988; van Gestel et al., 1998; Winklbauer, 1990; Winklbauer et al., 1992). Proper regulation of the actin cytoskeleton may prevent damage to these tissues and better enable these movements to occur (Gerthoffer, 2005; Li et al., 2005). Additionally, cell spreading induced by LPA signaling may allow for more surface area between two cells and increase cell-cell contacts to ensure that tissues are not ripped apart by these forces and that morphogenesis is completed successfully.

**Redundancy: Is an LPA signal the same if it comes from XLPA\textsubscript{1} or XLPA\textsubscript{2}?**

This work shows that both LPA receptors are necessary and sufficient to regulate actin density at the blastula stage, but XLPA\textsubscript{1} functions as a survival and proliferation signal in the anterior CNS in later development and this is not
rescued by XLPA$_2$. This demonstrates that the receptors have distinct roles, which may or may not be redundant. Several data support redundant roles for the receptors controlling actin dynamics. First, when XLPA$_1$ is depleted by an antisense phosphorothioate oligo and animal caps are excised early, a robust depletion phenotype is seen. Second, when the XLPA$_1$-depleted embryos are allowed to develop to just prior to gastrulation (Note: at room temperature stage 9 lasts for roughly three hours), then an actin phenotype is either very weak or absent. Zygotic XLPA$_1$, XLPA$_2$ mRNA, or both rescues the maternal XLPA$_1$ depletion. Third, when XLPA$_2$ is depleted by a MO approach in the embryo, no actin phenotype is apparent if animal caps are removed at the early blastula stage; however, when the caps are excised from these embryos prior to gastrulation then a phenotype is seen.

Further evidence for redundancy comes from double depletions and rescue experiments. When oocytes are depleted first by antisense oligos against maternal XLPA$_1$ and further depleted by injecting the XLPA$_2$ MO into the embryo, a more severe actin phenotype is seen. Moreover, in an XLPA$_1$ depletion the phenotype may be rescued by injecting back either XLPA$_1$ or XLPA$_2$ mRNA into the embryo and vice versa.

Downstream of both LPA receptors the same intracellular pathways are activated to influence cellular architecture (activation of both Rho and Rac). In cell culture and MEFs, these pathways have been delineated as well as in this work (Contos et al., 2002; Fang et al., 2000; Fukushima et al., 1998; Fukushima et al., 2000; Goetzl et al., 1999; Hama et al., 2004; Ishii et al., 2000). Therefore, it
is not surprising that the receptors may compensate for each other in terms of actin dynamics.

This redundancy may explain why only one maternal LPA receptor is present in *Xenopus*. The primary roles of LPA signaling up to the blastula stage may be to regulate the density of the actin cytoskeleton, allow cells to function as a tissue, and to protect against wounding. These functions would require one LPA receptor, but the other receptor would be able to compensate for the same functions. In fact, XLPA$_1$ mRNA is expressed at high levels in the oocyte, but after MBT levels fall dramatically while XLPA$_2$ mRNA levels increase throughout the embryo. The increase in XLPA$_2$ mRNA may be enough to prevent changes in actin due to decreased XLPA$_1$. Additionally, the onset of XLPA$_2$ mRNA expression begins just before gastrulation, when germ layers will be separated and tissues will begin to differentiate. Zygotic expression of both LPA receptors will prepare the embryo for separate roles for the two receptors.

Though most of the intracellular pathways activated by these two receptors are similar, there may be critical differences at later stages in different cell types. One response that differs between the two receptors is the ability of XLPA$_1$ to inhibit adenylate cyclase accumulation. This may be a critical difference between the two receptors that leads to a dramatic response with XLPA$_1$. There are few data showing if the LPA receptors have higher affinities for one class of G proteins compared to another. It is possible that in the anterior CNS, XLPA$_1$ is in close association with complexes containing G$_{i/o}$ proteins or preferentially activates this class of G protein. Upon receipt of an LPA signal, the receptor
would activate primarily survival and proliferation pathways compared to cytoskeletal pathways. XLPA$_2$ may have a higher affinity or be clustered with G$_q$ or G$_{12/13}$ families and thus mediate different effects. In fact, the most diversity between XLPA$_1$ and XLPA$_2$ lies in the C-terminus and this region is likely to mediate key interactions with intracellular protein signaling complexes.

Finally, S1P$_1$ mRNA expression also begins around the onset of gastrulation and this may influence actin dynamics. S1P$_1$ activates different downstream pathways than do LPA receptors, primarily G$_{i/o}$ pathways, and thus influences mainly cell migration, proliferation, and survival as opposed to cell architecture and morphology (Hla, 2004; Saba and Hla, 2004). Addition of S1P to animal caps did not result in either an increase in cortical actin or faster cap rounding like LPA. Also, overexpression of S1P$_1$ or its depletion by MO did not affect wound healing or actin dynamics. To show that there is no redundancy between S1P$_1$ and the LPA receptors, the LPA loss-of-function phenotype could not be rescued by reintroduction of the S1P$_1$ receptor. If S1P$_1$ is redundant, it should be able to rescue this phenotype. Therefore, LPA receptors are redundant in their ability to regulate actin density at the blastula stage, while S1P does not appear to have these effects.

**Other pathways activated by LPA receptors during the blastula stage**

Though we have shown that Rho and Rac are necessary for the overexpression effects of LPA receptors, it is possible that other pathways are activated in response to LPA during a wounding response. LPA is able to
influence behaviors, such as proliferation and cell survival, at later stages and these responses are critical during a wounding response. In the future, these pathways may also be examined in the animal cap assay to see if LPA prevents cell death after injury. Thus the overall LPA response after wounding would be to prevent further damage and initiate repair through rapid wound closure so that inner cells are not exposed to the outer environment, to promote cell survival, and to increase cell proliferation and aid in repopulation of the missing tissue.

**Differential roles for LPA receptors are uncovered by targeting their depletion at later stages.**

Here we show that XLPA$_1$ is required to maintain cell survival and proliferation in the anterior CNS, but not in either the posterior CNS or in the epidermis. Although a dorsal, animal injection of the XLPA$_1$ MO at the 8-cell stage targets all of the developing CNS on one side of the embryo, the apoptosis phenotype is seen only in the anterior CNS (defined as far posterior as the hindbrain-spinal cord junction). In addition, injection of the XLPA$_1$ MO into a ventral, animal cell does not increase levels of apoptosis within the developing epidermis. Other factors may control cell survival in these regions or survival signals may occur through both XLPA$_1$ and other receptors.

One possibility is that LPA signaling is a survival and proliferative signal for undifferentiated nervous tissues with an anterior identity, while other factors maintain tissues that are in a more differentiated state. Both the epidermis and posterior CNS begin to differentiate much earlier than the anterior CNS and this
may contribute to their lack of responsiveness to an LPA signal. When the epidermis and posterior CNS are induced originally, they are in an undifferentiated state. During this period, they may not respond yet to LPA signals or the lack of LPA signaling may not be sufficient to kill these tissues. In the anterior CNS after neural induction is completed, this tissue may begin to respond to LPA survival signals during maintenance of neural fate and thus rely heavily on LPA at this stage.

Most of the apoptosis and reduced proliferation in XLPA₁-depleted embryos is detected at the end of neurulation. This is after initial neural induction and most of the posterior CNS, as well as the epidermis, has begun to differentiate. The anterior CNS, on the other hand, still contains a large amount of undifferentiated tissue. Thus, there could be an early survival signal during the process of neural induction (Fig. 3), and at later stages of neurulation, LPA signaling may be critical for survival and proliferation.

Injection of the XLPA₁ MO into a ventral, animal cell does lead to defects at later stages. The most notable defect is a lack of pigmentation over the dorsal aspect of the head and gut regions. It is possible that neural crest may not be properly induced, neural crest cells may be unable to delaminate and migrate from the neural tube, neural crest may be unable to migrate over tissues lacking LPA signaling, or neural crest cells may rely on LPA signals for proliferation and cell survival. The assays used to detect the deteriorating CNS may not be sensitive enough to differentiate between dying neural crest cells and dying neural tissue, especially if the neural crest have not migrated from the neural
tube. Further exploration of this phenotype, by targeting the XLPA\textsubscript{1} to a ventral, animal cell, coupled with transplantation experiments may uncover further roles for LPA signaling in other germ layers. LPA signaling may be required in the neural crest cells or in the mesoderm or epidermis to regulate chemotaxis and migration. Using transplant experiments, labeled neural crest cells that are depleted of XLPA\textsubscript{1} may be transferred into a wild-type background and vice versa. This will help to address where the receptor is required and how it may function.

Phenotypes may not be present in the epidermis if XLPA\textsubscript{2} is redundant in this tissue. In cell culture, XLPA\textsubscript{1} activates similar survival and proliferation pathways as XLPA\textsubscript{2}. Both receptors may be necessary in other tissues and thus double depletions would have to be performed to uncover roles here. This is not as likely since both LPA receptors are expressed in the epidermis and in all of the CNS. If there is redundancy, then downstream regulation may determine why
only one receptor is required in the anterior CNS, but both can function in the epidermis.

**How does an LPA signal protect against or modulate apoptosis and stimulate cell proliferation?**

A likely downstream candidate to execute the response to an XLPA₁ signal is the c-Jun N-terminal kinase (JNK), a member of the MAPK family. JNK is an intracellular mediator of extracellular stresses on the cell. JNK is activated by growth factor withdrawal, with cytokine release, and with changes in environmental conditions, such as pH changes. Signals may be integrated at the level of JNK activation and cell proliferation, differentiation, and death pathways may be activated. Regulation of JNK signaling outcomes is dependent highly on scaffolding proteins. Scaffolds control both the temporal and spatial activation of JNK within the cell and regulate how the responses will be carried out. Recruitment of JNK and upstream activators are critical for the proper readout (Morrison and Davis, 2003).

GPCRs activate JNK primarily through the recruitment of β-arrestin to the receptor. Upon ligand binding to the GPCR, the receptor is phosphorylated and β-arrestin controls the internalization of the receptor. β-arrestin recruits members of the JNK cascade and activates cell proliferation mechanisms. In mammals, both β-arrestin2 and JNK3 are expressed highly in both the heart and brain and these two proteins have a high affinity for each other. Together JNK and
scaffolds may be regulated coordinately to control cell proliferation in response to a GPCR signal (Morrison and Davis, 2003).

Recently, the role for another JNK cascade scaffolding protein has been described in *Xenopus*. Plenty-of-SH3s (POSH) is a scaffolding protein that recruits JNK cascade members in response to activated Rac1 to promote NF-κB activation and eventually apoptosis (Tapon et al., 1998). Depletion of XPOSH in the anterior CNS using MO resulted in decreased levels of apoptosis in the anterior CNS (Kim et al., 2005). It is possible that recruitment of JNK to specific scaffolds is critical for proper neurogenesis. A balance between survival signals, such as LPA, that recruit JNK toward scaffolds that promote proliferation and prevent apoptosis, and stress signals that recruit JNK to XPOSH may be essential to ensure that the proper number of neurons form within the CNS.

**The ability of LPA to induce and influence gene expression: G_q pathways.**

This work does not address the ability of LPA to regulate gene expression directly in any of the phenotypes examined. In embryos depleted of XLPA_1_, gene expression is decreased in the anterior CNS, but it is unclear whether this is due to an LPA signal to maintain gene expression within the differentiating CNS or if the cells are dying and not proliferating and thus unable to respond to other signals. This may be addressed by blocking the apoptosis phenotype and then examining gene expression patterns. Recently, apoptosis was blocked successfully in the developing *Xenopus* CNS by overexpression of the human form of *bcl2* (Yeo and Gautier, 2005). Using similar techniques, the apoptosis
seen in the XLPA₁-depletion may be blocked by coinjection of mRNA for anti-apoptotic factors and then assaying for changes in gene expression. If gene expression is still absent within the anterior CNS in the coinjection, it suggests that XLPA₁ also plays a role in the maintenance of these genes by influencing gene expression. If gene expression is restored, then it implicates XLPA₁ only as a cell survival and proliferation signal.

**Future directions with lipid receptors**

As these data are translated to mammalian systems, it is possible that cells will contain a “lipidome” – a signature profile based on which lipid receptors are expressed on the cell surface that dictates how the cell will react to a lipid signal. The overall integration of the pathways based on which lipid receptors are expressed will then be translated into specific responses that differ between cell types. Most mammalian cells express at least two LPA and two S1P receptors (Anliker and Chun, 2004). For example, cells in the ventricular zone of the nervous system may express LPA₁, LPA₂, S1P₁, and S1P₃ and this may enable them to respond to lipid signals as proliferation and survival signals, while ovarian cancer cells may upregulate LPA₁, LPA₃, S1P₂, and S1P₄ and respond to lipids as chemoattractants and for cell proliferation to aid in invasion and metastasis (Fig. 4). Phenotypes in each tissue will only be apparent when the appropriate combination of receptors is deleted in mammals.
CONCLUSIONS

This work demonstrates that *Xenopus* LPA receptors have both overlapping and distinct roles throughout early development, not previously recognized in other organisms. During the blastula stage, signaling through LPA receptors is critical for modulating the density of the actin cytoskeleton and during wound healing. At post-gastrula stages, a unique role for XLPA₁ is to serve as a survival and proliferation signal in the anterior CNS. It will be interesting to determine the roles of these lipid receptors in other tissues throughout development. An unanswered question is how is lipid signaling controlled? Is it by production of the ligand, termination of lipid signals, or via a combination of these mechanisms? Additionally, determining the different stimuli
for lipid production may better uncover how this type of signaling is regulated during development.
References


Appendix
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* XLPₐ₁ 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 pseudooallele have so far been identified. These are most closely related to mammalian LPA1 (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 using 1 mM progesterone and fertilized using the host transfer technique as reported previously (Holwill et al., 1987). Embryos were dejellied in a 2% cysteine/0.1× MMR solution (pH=7.8) and maintained in 0.1× MMR. Embryo stages cited are as described by Nieuwkoop and Faber (1967). For mRNA and morpholino injections, embryos were dejellied in Ca2+/Mg2+-free MMR on a 1% agarose dish. After 1 hour, cells were removed from the LPA solutions and maintained in Ca2+/Mg2+-free MMR in glass dishes for five minutes, or allowed to reassociate in Ca2+/Mg2+-free MMR. Cells were transferred into 0.1-1 mM LPA in Ca2+/Mg2+-free MMR in glass dishes for five minutes, or allowed to reassociate in Ca2+/Mg2+-free MMR. Cells were incubated for 10 minutes in a lipid or control solution before analysis of cortical actin.

Oligonucleotides

Twelve antisense oligonucleotides complementary to both XLPA1 and XLPA2 mRNA were tested for their ability to deplete the maternal messages 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-GTAGTAGCAC*T*G*G 3′.

Morpholino oligonucleotides were designed that targeted both XLPA1 and XLPA2. These were resuspended in sterile, filtered water and injected at doses of 10-40 ng into either oocytes or embryos: XLPA1A and XLPA2. These were resuspended in sterile, filtered water and injected at doses of 10-40 ng into either oocytes or embryos: XLPA1A and XLPA2. These were resuspended in sterile, filtered water and injected at doses of 10-40 ng into either oocytes or embryos: XLPA1A and XLPA2.

RNA constructs

Clones encoding both X. tropicalis XLPA1 and XLPA2 were identified by blasting the murine sequences for LPA1 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 Apol. 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 and look for the formation of primer dimers. Primer pairs that were used are as follows.

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**XLPA1:**

- F: 5′ CTTGGAGTCCCGTGTGTTTT 3′
- R: 5′ TGGCT-GCAGAAGTCTGTGAC 3′

**XLPA2:**

- F: 5′ TTCTTCTGCAACAGGGGTTC 3′
- R: 5′ GGGCCT-CACCTCACAAGTTCTTTT 3′

**ODC:**

- F: 5′ GCC ATT GTG AAG ACT CTC TCC ATT C 3′
- R: 5′ TTC GGG TGA TTC CTT GCC AC 3′

**Plakoglobin:**

- F: 5′ GCT CGC TGT TCA ACC AGC ATT C 3′
- R: 5′ GTA GTT CCT CAT GAT CTC AAC C 3′

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 (Snake 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 0.1-0.1 mM LPA in Ca2+/Mg2+-free MMR in glass dishes for five minutes, or allowed to reassociate in 1× MMR. Cells were removed from 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× 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 µm\(^2\) 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 µm\(^2\) 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× 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× 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 LPA₁ receptor. The sequences for murine LPA₂ and LPA₃ 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 LPA₂. 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* XLPA₂ mRNA was deposited into GenBank as Accession Number AY652941.

The predicted protein was found to be 62% identical and 16% similar to mouse LPA₂ at the protein level and thus was designated *X. tropicalis* XLPA₂. 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). XLPA₂ 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). XLPA₂ 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 LPA₂ receptors, it also lacks the longer extracellular N terminus of LPA₁.

No orthologs of mouse LPA₃ 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 XLPA₁ and XLPA₂ analyzed by real-time RTPCR. As reported previously, XLPA₁ 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 XLPA₁ transcript fall, but low levels of XLPA₁ expression continued until at least stage 45.

Conversely, XLPA₂ 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 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 XLPA₁ or XLPA₂ 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 XLPA₁ or XLPA₂ 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 mm² area from 1133±177 to 1372±302 or 1610±348 for XLPA₁ and XLPA₂, 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 LPA₁ 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 LPA₁ mRNA using antisense oligodeoxynucleotides. Twelve oligos, each complementary to both XLPA₁A and XLPA₁B, 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 16 hours later by the host transfer technique (Holwill, 1987).

Injection of both morpholino oligos together caused a generalized decrease in the amount of F-actin staining from 1122±87 to 1002±59 (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 higher magnification, actin filament bundles in the cell cortices were dramatically reduced in density, compared with the controls (Fig. 7D). The formation of actin-rich filopodia in the early blastula embryos was unaffected in these embryos. Neither the overexpression nor depletion of LPA receptors affected the number or size of contractile rings seen in dividing cells. The caps contained the normal number of cells and there was no evidence of undividing cells in these embryos (data not shown). The effects of the thioate oligo were reversed (Fig. 7C) by depleting XLPA1, fertilizing the oocytes by host transfer and injecting 400 pg of XLPA1 mRNA at the two-cell stage, by which time the antisense oligo had degraded (Raats et al., 1997).

The average mean intensity of phalloidin staining decreased from 1532±395 to 978±202 and 877±191 for the 7.5 ng and 10 ng dose of XLPA1-10MP, respectively (Fig. 4C). 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.

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 morpholinos reduced the average mean intensity of phalloidin staining from 1122±87 to 1002±59 (Fig. 4D). These data suggest that after the mid-blastula stage, the combined levels of the two LPA receptors are necessary to maintain the pattern and density of cortical actin in the embryo. In contrast to removal of only the maternal LPA1 mRNA,
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 purse 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 XLPA1 and XLPA2 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 XLPA1. This was most likely due to the onset of XLPA1 and XLPA2 production after the MBT. Redundancy also exists between murine LPA receptors. The Edg4–/– mouse (mouse homologs of LPA1 and LPA2 are known as Edg2 and Edg4, respectively) showed no obvious gross or histological phenotype and the Edg2+/−/Edg4−/− mouse only showed an increase in frontal hematomas compared with the Edg2−/− mouse (Contos et al., 2002). In addition, when LPA was added to mouse embryonic fibroblasts isolated from the mepharinos, stress fibers formed throughout the cell. This response was only blocked in fibroblasts isolated from the Edg2−/−/Edg4−/− 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 LPA1 and LPA2 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

![Fig. 8. Dominant-negative forms of RhoA and Rac1 block the overexpression effects of XLPA2.](image)

(A) Low-power magnification. Scale bar: 20 µm. (Left panels) A control cap (upper) and a cap injected with 100 pg of XLPA2 mRNA (lower) into each cell at the two-cell stage. (Upper middle) Overexpression of RhoA-N19 blocks purse-string formation and delays wound healing with no change on cortical actin. (Lower middle) The overexpression effects of XLPA2 on wound healing are blocked by RhoA-N19, but not the increase in cortical actin. (Right panels) Rac-N17 also prevents purse-string assembly and reduces the amount of cortical actin (upper) and blocks the effects of XLPA2 (lower). (B) High-power magnification. Scale bar: 5 µm. (Upper left) Cellular network in a cap injected with a low dose of XLPA2 mRNA. (Lower left) Injection of RhoA-N19 results in an increase in cortical processes and (upper middle) prevents the formation of an actin purse-string (arrow). (Upper right) Cells in caps injected with both XLPA2 and RhoA-N19 still have cell processes similar to RhoA-N19 alone and no purse-string. (Lower middle) Rac-N17 caused a decrease in the amount of cortical actin, decreased the number of cell processes, and caused the cells to become rounded. (Lower right) When co-injected with XLPA2, Rac-N17 blocks the increases in cortical actin and formation of rigid network, but cell processes are still evident.
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 $G_{\alpha 12/13}$ 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 $G_{\alpha i/o}$-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, 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 actinomyosin 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


Wood, W., Jacinto, A., Grose, R., Woolner, S., Gale, J., Wilson, C. and


