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Two Novel *Xenopus* Homologs of Mammalian LP_{A1}/EDG-2 Function as Lysophosphatidic Acid Receptors in *Xenopus* Oocytes and Mammalian Cells*

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Lysophosphatidic acid (LPA) induces diverse biological responses in many types of cells and tissues by activating its specific G protein-coupled receptors (GPCRs). Previously, three cognate LPA GPCRs (LP_{A1}/VZG-1/EDG-2, LP_{A2}/EDG-4, and LP_{A3}/EDG-7) were identified in mammals. By contrast, an unrelated GPCR, PSP24, was reported to be a high affinity LPA receptor in *Xenopus laevis* oocytes, raising the possibility that *Xenopus* uses a very different form of LPA signaling. Toward addressing this issue, we report two novel *Xenopus* genes, *xlp_{A1}-1* and *xlp_{A1}-2*, encoding LP_{A1} homologs (~90% amino acid sequence identity with mammalian LP_{A1}). Both *xlp_{A1}-1* and *xlp_{A1}-2* are expressed in oocytes and the nervous system. Overexpression of either gene in oocytes potentiated LPA-induced oscillatory chloride ion currents through a pertussis toxin-insensitive pathway. Injection of antisense oligonucleotides designed to inhibit *xlp_{A1}-1* and *xlp_{A1}-2* expression in oocytes eliminated their endogenous response to LPA. Furthermore, retrovirus-mediated heterologous expression of *xlp_{A1}-1* or *xlp_{A1}-2* in B103 rat neuroblastoma cells that are unresponsive to LPA conferred LPA-induced cell rounding and adenylyl cyclase inhibition. These results indicate that XLP_{A1}-1 and XLP_{A1}-2 are functional *Xenopus* LPA receptors and demonstrate the evolutionary conservation of LPA signaling over a range of vertebrate phylogeny.

Lysophosphatidic acid (LPA[1]; 1-acyl-2-*sn*-glycerol-3-phosphate) is a simple phospholipid that exerts hormone- and growth factor-like effects in many organisms and organ systems. LPA can alter cell fates by inducing proliferation and differentiation or by preventing apoptosis in many cell types (1). In addition, LPA can induce cytoskeletal reorganization that leads to cell rounding and stress fiber formation (1–3).

Biological responses to LPA are elicited by activation of its specific G protein-coupled receptors (GPCRs). Thus far, three genes (lp_{A1}, lp_{A2}, and lp_{A3}) encoding high affinity LPA receptors, LP_{A1}/EDG-2, LP_{A2}/EDG-4, and LP_{A3}/EDG-7, have been identified in mammals (reviewed in Refs. 3 and 4). Biological functions of these receptors have been characterized by overexpression and/or heterologous expression in mammalian cells (5–13). All three LPA receptors can mediate adenylyl cyclase inhibition, increases in intracellular calcium, inositol phosphate production, and MAP kinase activation. LP_{A1} and LP_{A2} can also induce cell rounding via activation of the small GTPase, Rho. Pharmacological studies suggest that both LP_{A1} and LP_{A2} couple to at least three types of G proteins, $G_{i/o}$, $G_{12/13}$, and G_q, whereas LP_{A3} couples with $G_{i/o}$ and G_q but not with $G_{12/13}$ (13). Genetic deletion of LP_{A1} in mice demonstrated that LP_{A1} is at least in part responsible for LPA signaling *in vivo* and is essential for normal development (14).

A molecularly different LPA receptor was reported in studies on *Xenopus* oocytes (15). Guo *et al.* (15) isolated a novel GPCR gene, *PSP24*, by polymerase chain reaction (PCR) using degenerate oligonucleotide primers against a platelet-activating factor receptor. Overexpression of PSP24 in oocytes potentiated maximal LPA-induced oscillatory chloride ion (Cl^-) currents, whereas injection of antisense oligonucleotide against PSP24 inhibited endogenous responses to LPA. Based on these observations, the authors concluded that PSP24 is a high affinity receptor for LPA. However, subsequent studies by others were inconsistent with this conclusion. Heterologous expression of PSP24 did not mediate LPA responses in yeast, whereas LP_{A1} did (7). In addition, mammalian orthologs of PSP24 did not mediate responses to LPA in assays such as [^{35}S]GTPγS binding, [^{3}H]LPA binding, MAP kinase activation, [^{3}H]thymidine incorporation, adenylyl cyclase inhibition, and increases in intracellular calcium (16, 17).

Mouse, human, and *Xenopus* homologs of PSP24 share ≥55% amino acid sequence identity with one another (18). These PSP24s have comparatively little amino acid sequence identity (≤20%) with members of the mammalian LP_A receptor family (4). They instead show closest similarity to receptors for a bioactive peptide, cholecystokinin (4). The dissimilarity between mammalian and *Xenopus* LPA receptors was surprising based on the phylogenetic conservation of many other GPCRs

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The nucleotide sequences reported in this paper have been submitted to the EMBL/GenBank™/EBI Data Bank with accession numbers AJ249843 and AJ249844.

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[1] The abbreviations used are: LPA, lysophosphatidic acid; GPCR, G protein-coupled receptor; PCR, polymerase chain reaction; GTPγS, guanosine 5′-*O*-(thiotriphosphate); PTX, pertussis toxin; GFP, green fluorescent protein; EGFP, enhanced GFP; TMD, transmembrane domain(s); bp, base pair(s); ORF, open reading frame; kb, kilobase(s); Y, cytidine or thymidine; W, adenosine or thymidine; S, guanosine or cytidine; R, adenosine or guanosine.

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for a single ligand. This disparity raises the question of whether *Xenopus* might use a fundamentally different LPA receptor system or might also express and use LPA receptors homologous to those found in mammals. Here, we report identification and characterization of two novel *Xenopus* GPCRs that show 89–90% amino acid sequence identity with mammalian LP_{A1}.

EXPERIMENTAL PROCEDURES

Materials—[α-^{32}P]deoxy CTP was purchased from PerkinElmer Life Sciences. LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, AL). Pertussis toxin (PTX) was purchased from Calbiochem (La Jolla, CA). B103 rat neuroblastoma cells (19) were a gift from Dr. David Schubert (The Salk Institute, La Jolla, CA). RH7777 rat hepatoma cells were a gift from Dr. Hyam Leffert (University of California, San Diego, La Jolla, CA). Retrovirus expression vector (LZRS-EGFP) and Phoenix ecotropic retrovirus producer cell lines were gifts from Dr. Garry P. Nolan (Stanford University, Stanford, CA). Y-27632 was a gift from Welfide Pharmaceutical Industries (Saitama, Japan). Trizol and all cell culture reagents were purchased from Life Technologies, Inc. Anti-GFP antibody was obtained from CLONTECH (Palo Alto, CA). Forskolin, 3-isobutyl-1-methylxanthine, anti-FLAG M2 monoclonal antibody, and other reagents were purchased from Sigma, unless otherwise noted.

Amplification of Xenopus cDNAs by Reverse Transcription-PCR—mRNA was prepared from *Xenopus* oocytes using the Oligotex direct mRNA kit (Qiagen, Valentia, CA) as described by Ferby *et al.* (20). First strand cDNA was synthesized from 500 ng of mRNA using oligo(dT) primers and the SUPERSCRIPT first-strand synthesis system (Life Technologies, Inc.). This cDNA was used as a template for PCR with degenerate primers designed toward sequences in transmembrane domains (TMD) II and VII conserved among members of the GPCR family (21). The nucleotide sequence for the TMD II primer is 5′-CCIATGTAYYTIT-TYYTYWSGAATTCIWSITTI-3′, and the sequence for the TMD VII primer is 5′-AARTCIGGRSWICGISARTAIATSAIIGGRTT-3′. The PCR condition was 40 cycles of 94 °C for 1 min, 45 °C for 1.5 min, and 72 °C for 2 min. After electrophoresis on agarose gels, three prominent bands of the expected size range (400–1300 base pairs (bp)) were recovered from the gel and re-amplified by PCR under the same conditions. The final PCR products were cloned into pCR 2.1 using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced.

Cloning of Full-length Xenopus cDNAs—To obtain full-length cDNAs for the PCR products, we screened a *Xenopus* oocyte cDNA library (a gift from Dr. John Shuttleworth, University of Birmingham, UK) using a ^{32}P-labeled 560-bp PCR fragment as a probe. Two different full-length *Xenopus* LPA receptor isoforms were isolated as XLPAR-1 and XLPAR-10. XLPAR-1 contains 2053 bp, with 321 bp of 5′ untranslated region and 631 bp of 3′ untranslated region followed by a poly(A) tail. XLPAR-10 contains 1941 bp and lacks a poly(A) tail. These cDNA sequences were deposited in the EMBL data base with the accession numbers AJ249843 (XLPAR-1) and AJ249844 (XLPAR-10). In view of their high degree of homology to mammalian LP_{A1}, they are referred to here as XLP_{A1}-1 and XLP_{A1}-2 for XLPAR-1 and XLPAR-10, respectively. Nucleic acid and amino acid alignment was performed using the Clustal W multiple sequence alignment program found on the Web page of the DNA Data Bank of Japan.

Northern Blot Analysis—Tissues were quickly removed from female *Xenopus*, and total RNA was isolated from each tissue using Trizol according to the manufacturer's instructions (Life Technologies, Inc.). Northern blotting was performed as described previously (6, 13), and membranes were analyzed with a Bio-Imaging analyzer BAS2500.

Electrophysiology in Xenopus Oocytes—Open reading frames (ORFs) of *xlp_{A1}-1* and *-2* were subcloned into *Bam*HI-*Xho*I sites of the pBluescript SK(+) vector (Stratagene). Constructs were linearized with *Kpn*I digestion and used as a template in *in vitro* RNA transcription using mMESSAGE mMACHINE Kits (Ambion, Austin, TX). *Xenopus* oocyte preparation, cRNA injection, and electrophysiology were performed as described previously (22). Briefly, stage V and VI oocytes from adult females were injected with 50 nl of appropriate cRNA (1 μg/μl) for overexpression and incubated at 16 °C for 3–5 days in modified Barth saline (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM Hepes (pH 7.4)) before recording. Oocytes were impaled by two microelectrodes filled with 3 M KCl and voltage-clamped at −50 mV. Only oocytes with resting potentials of less than −30 mV were used. Oocytes were continuously superfused with Ringer's solution (120 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 50 mM Hepes (pH 7.4), 0.1% (w/v) fatty acid-free bovine serum albumin) in the presence of LPA. For antisense oligonucleotide studies, injection of oocytes with 50 nl of oligonucleotides (2 μg/μl) was performed 3–5 days before recording. The antisense oligonucleotides were designed to complement 11–12 nucleotides 5′ and 3′ to the initiation codon for *xlp_{A1}-1* or *xlp_{A1}-2*. All oligonucleotides were phosphorothioated near 5′ and 3′ ends (*) to prevent degradation (23). Antisense oligonucleotide sequences were 5′-G*A*A*A*GAGAAGCCAUUUUAGC*C*C*A*G-3′ for *xlp_{A1}-1* and 5′-G*A*A*A*GCGAAGUCAUUUUAG*C*C*C*A*G-3′ for *xlp_{A1}-2*. Because the antisense oligonucleotides differed by only two nucleic acids, a single sense-orientation oligonucleotide corresponding to the region targeted for *xlp_{A1}-1* and *-2* was used as a negative control (5′-C*U*G-*G*GCUAAAAUGGCUUCGC*U*U*U*C-3′). For the PTX experiments, *Xenopus* oocytes were incubated with 2 μg/ml PTX in modified Barth saline for 48 h before recording.

Retrovirus Systems—The entire ORFs for *xlp_{A1}-1* and *-2* were subcloned into *Hin*dIII and *Xba*I sites of a pFLAG-CMV-1 mammalian expression vector (Eastman Kodak Co.) to introduce preprotrypsin-leader/FLAG-tag sequences into amino-terminal extracellular regions of each receptor for immunocytochemical detection of the receptor proteins. These constructs were then subcloned into *Bam*HI and *Xho*I sites of a Moloney murine leukemia retroviral vector, LZRS-EGFP (24). Sequences of internal ribosomal entry sites in the vector enable concomitant expression of EGFP and FLAG-tagged receptors within the single cell (13). The inserts of the constructs were confirmed by sequencing. Retrovirus supernatants were prepared using a Phoenix cell line, as described previously (13).

Functional Assays—For the cell rounding assay, B103 cells were seeded onto glass coverslips coated with Cell-Tak (Becton Dickinson Labware, Bedford, MA) and infected with viral supernatants (13). After treatment with LPA, cells were fixed with 4% (w/v) paraformaldehyde and incubated with the blocking solution (0.1% (w/v) Triton X-100, 0.25% (w/v) bovine serum albumin in phosphate-buffered saline). EGFP protein was visualized by incubation with anti-GFP polyclonal antibody, followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA). FLAG-tagged receptor was visualized by incubating cells with anti-FLAG antibody, followed by incubation with Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were observed with a Zeiss Axiophot and a Plan-Neofluor × 40 objective (Carl Zeiss, Thornwood, NY) or a confocal laser-scanning microscope TCS NT and a PL APO 63 × 1.20 water-immersion objective (Leica, Deerfield, IL). For stress fiber formation assays, fixed RH7777 cells were immunostained for FLAG and polymerized actin, as previously described (5). For measurement of intracellular cAMP contents, retrovirus-infected B103 cells were stimulated with LPA in the presence of 1 μM forskolin and 0.5 mM 3-isobutyl-1-methylxanthine. Intracellular cAMP contents were measured using a cAMP enzyme-immunoassay system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Statistical Analysis—Data shown are the means ± S.E. from replicate samples from replicate experiments. Statistical analysis was performed by Student's *t* test.

RESULTS

Isolation of xlp_{A1}-1 and xlp_{A1}-2—To identify novel GPCRs in *Xenopus* oocytes, we performed a PCR-based screen using degenerate oligonucleotide primers designed against TMD II and VII (6, 21). The PCR amplifications resulted in three faint bands in the expected size range for the region between TMD II and TMD VII of GPCRs (400–1300 bp) that were re-amplified and cloned. DNA sequencing identified two fragments with 90% identity in predicted amino acid sequences to those of the mammalian LP_{A1} receptor. These PCR fragments were then used as probes to screen a *Xenopus* oocyte cDNA library, and two different cDNAs (*xlp_{A1}-1* and *xlp_{A1}-2*) were cloned, which encoded GPCRs consisting of 366 amino acids that differed by 6 amino acids (Fig. 1, *A* and *B*). The comparison of nucleic acid sequences showed that *xlp_{A1}-1* was 96% identical in the predicted ORF with *xlp_{A1}-2*, whereas there was much less identity in their 5′ and 3′ untranslated regions (69 and 61%, respectively; Fig. 1*A*). Because the nucleotides that differ between *xlp_{A1}-1* and *-2* are distributed throughout the entire ORFs, *xlp_{A1}-1* and *-2* were probably encoded by different genes rather than produced by alternative splicing.

A

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xlpA1-1  GAGCGTTTAGGGCATTACACTCAGCTGCCTGGGAGAAGCCGGTCTGTGCATGGTCTCCCGTGCCTCTATGGTACCTGT--------ACCG  -240
xlpA1-2  --------------------  C  C      T                  TA                         GTAACCCT  T  -274

xlpA1-1  CCCCCCTGTCCAGCACCCCTTTTCACAAACCTCGCAATTCGATTGCTAGCATTTAT----ATCTGCTAATTTGGATATTTGCTTCTCTC-  -155
xlpA1-2      T GC      T            G      G     T   CG  A  CA  CCTGG  ACTTA G       G C       A  A  -184

xlpA1-1  -----------------------GCTGAACCTCCGCTGTCGGCTTTTAGAGTT---TAAACGAGA---GAACCACAATGGAACTTGGAGT   -94
xlpA1-2  ACTGGATATCATTACTTACTCGC        CTTT   C       C R   GAGG G  A    TTA   T   C   A        A

xlpA1-1  CCCGTGTGTTTTATGGGTCACCAGCCAGCATGACTGACATCTGAAGTGAACTGGCACATATTGGAAACCTTTGATAAGACCTACTGGGCT    -4
xlpA1-2                       TT                                  G   C     T     T
            +1
xlpA1-1  AAAATGGCTTCTCTTTCAGAGTTTGTCAGTGAACCTATCAGTATGATGTCACAGACTTCTGCAGCCAGTGAATCCCAGTGCTACTACAAT    87
xlpA1-2        A    G                       C   G                         G

xlpA1-1  GAAACCATTGCCTTCTTTTACAACCGGAGTGGAAAATACCTAGCTACAGAATGGAATGCAGTCAGCAAGCTGGTGATGGGACTCGGAATC   177
xlpA1-2                                             A

xlpA1-1  ACAGTGTGTATATTCATCATGTTAGCAAACTTGCTGGTAATGGTGGCTATTTATGTCAACCGGCGGTTTCACTTTCCTATTTATTATTTA   267
xlpA1-2                            C

xlpA1-1  ATGGCCAACTTGGCCGCTGCAGACTTTTTTGCAGGACTAGCTTATTTTTACCTAATGTTTAATACAGGACCCAATACCAGAAGATTGACT   357
xlpA1-2                T                                                           C

xlpA1-1  GTAAGCACATGGTTGTTGAGACAGGGACTCATTGACACTAGCCTGACAGCGTCAGTAGCCAATTTGTTAGCCATTGCCATAGAAAGGCAC   447
xlpA1-2          G

xlpA1-1  ATAACAGTATTTCGAATGCAATTACACACCAGGATGAGCAACAGAAGAGTGGTAGTAGTGATTGTTGTTATCTGGACTGTGGCCATTGTC   537
xlpA1-2    T     T                                                        G

xlpA1-1  ATGGGAGCAATACCAAGCGTTGGATGGAACTGCATCTGTGATCTTGAGCAGTGCTCGAACATGGCTCCTCTTTACAGTGACTCCTATTTG   627
xlpA1-2             T     T                                C        T

xlpA1-1  ATATTTTGGACAATTTTCAATCTGGTTACTTTTGTAGTGATGGTAGTTCTCTACGCTCACATTTTTGTATATGTGCGACAGAAGACTATG   717
xlpA1-2    T  C              C                                                             G

xlpA1-1  AGAATGTCTAGGCACAGTTCTGGTCCTAGGAGGAATCGTGATACCATGATGAGCCTTCTGAAAACCGTGGTCATTGTTCTAGGTGCTTTT   807
xlpA1-2                         C              C     A

xlpA1-1  ATAGTTTGTTGGACTCCTGGCCTGGTGCTTTTGCTTCTTGACATCTGCTGTCCTCAGTGCAATATACTTGCTTATGAGAAGTTCTTTTTA   897
xlpA1-2                       T       C     A  A   G A     C  A

xlpA1-1  CTCCTGGCTGAATTCAACTCTGCTATGAACCCCATTATCTACTCCTATCGCGATAAGGAAATGAGTGCCACTTTCAAACAGATCCTTTGT   987
xlpA1-2                                                                               G

xlpA1-1  TGTCAGCGAACAGAGAATGTAAATGGACCCACGGAAGGTTCTGACCGCTCTGCTTCATCTCTCAACCATACTATATTGGCTGGTGTCCAT  1077
xlpA1-2                         C                                T        T

xlpA1-1  AGCAATGACCATTCTGTTGTTTGACAAGCGACAATACATTTCAGAAATAAGAGCTTTTGGGCAGAGAGGAGACCCCTTGCTTCATAGCGG  1167
xlpA1-2                   G                C             G A           AC              T   T     T

xlpA1-1  AAAGCGGCTC-GCAAACATTTGGACACAAGCTGTGATAATATCTAAAGAAATACACATATATTT----GAACACTAACCAGATAATCCAA  1252
xlpA1-2       T  CAA               G                                   AATCAG             C    T     1257

xlpA1-1  CCATTGCTGAAG------GAACTTATAGAAATAGTAGCAATATGAAATATGCTAGACTCTAAAAAA-------TCTGCATATGATACCTG  1329
xlpA1-2              TCCGAG   T    G     AC      C  A  G    AC    AT      CCGGCTTC        A          1347

xlpA1-1  TGTCAAATTTCCACATTTT-AACTGCC-------GTAGAGGCCTAGGTCTTTTTTTA-----------------AGATATATTCCATTT  1393
xlpA1-2         C           G       ATTTCAT                      TTTTTTCTTTTTTTTTTT  A     A     GA  1437

xlpA1-1  AACAAAACATACATTTTTAGTGCCATCATCATGCATGTGATGGACATTGGTTTGAAGTGACTTTATAACA-CTTGTGTAGCTGCACTGCC  1482
xlpA1-2    T               C  C      G                C   T                    A                   1527

xlpA1-1  TTAAAGTAAAAACAGAAAAATAAGAAATTTGATATTTTTCTAATATAAGCAAAAGAAATGTGTTAATTTTTGTGTGTAGTCTTTCTCCGT  1572
xlpA1-2             T   C                                                C       ------------------  1599

xlpA1-1  TATATGAAGTTCTATTATATATATATATAAATATATTCTTTTTTTAACTTTTTCTAGAGCAGAGGCATTTGTACAGTTTGAAGGAAACAA  1662
xlpA1-2  ------------------------------------------------------------------------------------------

xlpA1-1  AAAGGCCTTTAATAACCAATAGATAATAAAACAAAGAGGATGTTAATTTGTCAAAAAAAAAAAAAAAAAA                      1732
xlpA1-2  ----------------------------------------------------------------------
```

FIG. 1. **Comparative sequences of *xlp*$_{A1}$-1 and *xlp*$_{A1}$-2.** *A*, alignment of nucleotide sequences of *xlp*$_{A1}$*-1* and *xlp*$_{A1}$*-2* cDNAs. The lower sequence shows only those residues for *xlp*$_{A1}$*-2* that differ from *xlp*$_{A1}$*-1*. ORFs are indicated in *black boxes*. Gaps are indicated by -. *B*, alignment of amino acid sequences of XLP$_{A1}$-1, XLP$_{A1}$-2, mouse (*m*) LP$_{A1}$, and human (*h*) LP$_{A1}$. Putative TMD I–VII are *overlined*. Amino acid residues identical among all the sequences are indicated by *. Similar amino acid residues found in two or three sequences are indicated by :. Potential post-translational modification sites conserved among members of the GPCR family are also indicated: *N*-linked glycosylation sites (●), protein kinase C/casein kinase II phosphorylation sites with (S/T)*X*(R/K) motif (○), palmitoylation sites (X).

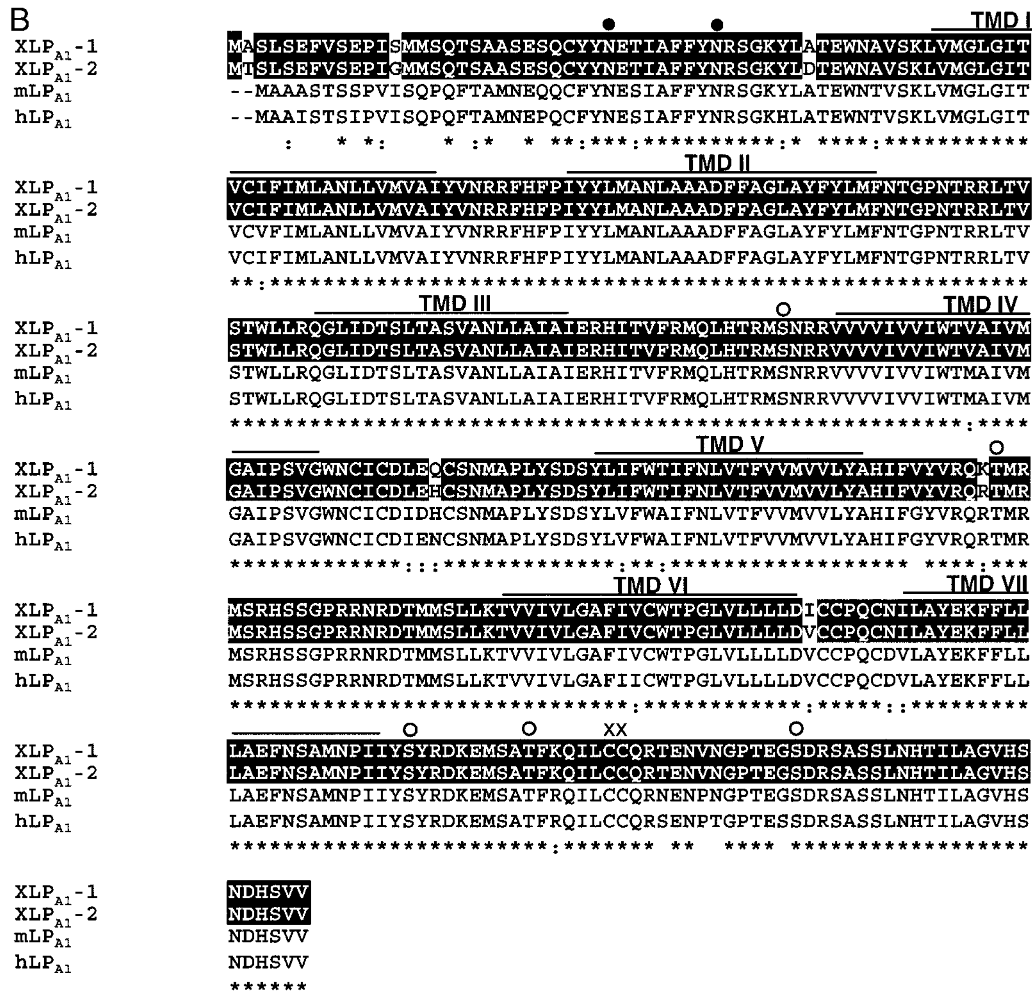

FIG. 1.—*continued*

Sequence alignment of these receptors with mouse and human LP_{A1} demonstrated high identity in both nucleic acid and predicted amino acid sequences. At the nucleic acid level, *xlp*$_{A1}$*-1* was 75% identical to mouse *lp*$_{A1}$ and 77% identical to human *lp*$_{A1}$, whereas *xlp*$_{A1}$*-2* was 78% identical to mouse *lp*$_{A1}$ and 77% identical to human *lp*$_{A1}$. A comparison of predicted amino acid sequences indicated that both clones were 89–90% identical to both mouse and human LP_{A1} (Fig. 1*B*). The amino acids were least conserved between *Xenopus* clones and mammalian LP_{A1}s in the first 24 amino acids of the amino-terminal regions (5 of 22 amino acids were identical).

Expression of xlp$_{A1}$*-1 and -2 in Xenopus Tissues*—To examine expression of *xlp*$_{A1}$*-1* and *-2* in *Xenopus*, total RNA from various tissues was isolated and analyzed by high stringency Northern blot analysis (Fig. 2). The strongest signal was observed in oocytes, where a band of ~2.2 kb was observed. In addition, brain and spinal cord samples expressed this 2.2-kb band but also expressed larger species of ~5.8 and 11 kb (Fig. 2). Because the nucleic acid sequences of *xlp*$_{A1}$*-1* differed by only 3% from *xlp*$_{A1}$*-2* and were dispersed throughout the ORFs, attempts to differentiate the forms by Northern blot analysis were unsuccessful. *xlp*$_{A1}$*-1* and *-2* expression was highest in oocytes, at lower levels in brain and spinal cord, and below detection in lung, heart, kidney, liver, muscle, stomach, and intestine.

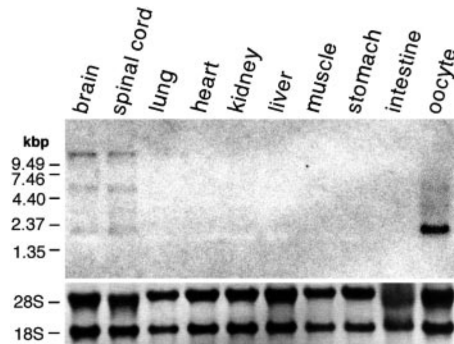

FIG. 2. **Expression of *xlp*$_{A1}$-1 and/or *xlp*$_{A1}$-2 in various *Xenopus* tissues.** Total RNA samples (10 μg) isolated from females were analyzed by high stringency Northern blot analyses as compared with a loading control (ribosomal RNA). Molecular size markers are indicated on the *left* in kb.

xlp$_{A1}$*-1 or xlp*$_{A1}$*-2 Overexpression in Xenopus Oocytes*—Application of LPA is known to evoke oscillatory inward Cl^- currents in native *Xenopus* oocytes, an indication that oocytes endogenously express LPA receptors (25–27). To examine whether XLP_{A1}-1 or XLP_{A1}-2 could function as high affinity LPA receptors, each was overexpressed by cRNA injection into oocytes, and the Cl^- currents in response to LPA were recorded (Figs. 3 and 4). Control (diethyl pyrocarbonate-treated water)-injected oocytes did not show any response to 3 nM LPA, whereas overexpression of either *xlp*$_{A1}$*-1* or *xlp*$_{A1}$*-2* elicited LPA-induced Cl^- currents at this concentration (data not shown). At higher LPA concentrations (10 nM), application of LPA on control oocytes induced small Cl^- currents averaging 50 nA (Figs. 3*A* and 4*A*). Overexpression of either *xlp*$_{A1}$*-1* or *xlp*$_{A1}$*-2* significantly potentiated the LPA-induced Cl^- currents

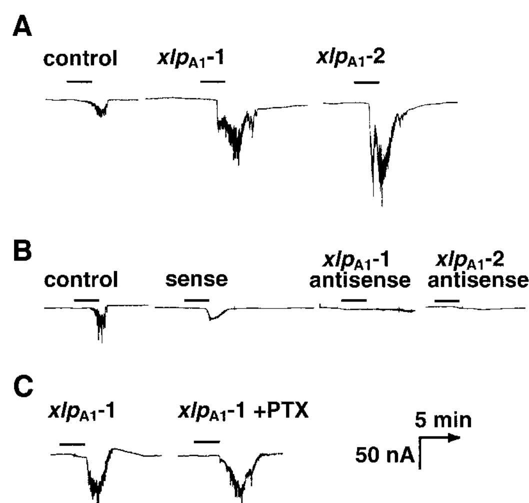

FIG. 3. **LPA-induced Cl⁻ currents in oocytes.** *A*, overexpression of XLP_{A1}-1 or XLP_{A1}-2. Overexpression of XLP_{A1}-1 or XLP_{A1}-2 produced by cRNA injection potentiates LPA (10 nM)-induced Cl^- current in *Xenopus* oocytes. *B*, oligonucleotide injection. Antisense oligonucleotides (100 ng/oocyte) designed against xlp_{A1}-*1* or xlp_{A1}-*2* inhibit endogenous responses to LPA, whereas injection of sense oligonucleotides did not alter LPA responses. *C*, effect of PTX treatment. PTX pretreatment does not inhibit LPA-induced Cl^- currents in xlp_{A1}-*1*-injected oocytes. A typical trace is shown in each panel.

(Figs. 3*A* and 4*A*). Sphingosine 1-phosphate, a structurally related bioactive lysophospholipid, did not evoke Cl^- currents in either control or xlp_{A1}-*1*- and/or xlp_{A1}-*2*-overexpressing oocytes (data not shown).

xlp_{A1}-1 or xlp_{A1}-2 Antisense Oligonucleotide Injection in Xenopus Oocytes—If XLP_{A1}-1 and/or XLP_{A1}-2 mediate endogenous LPA responses, a reduction in receptor expression could result in decreased Cl^- currents in response to LPA. To address this, sense (as a control) or antisense oligonucleotides were synthesized as 23–24-mers designed to block the initiation codon and thus inhibit xlp_{A1}-*1* and -*2* translation. When these oligonucleotides were injected into oocytes, only injection of antisense oligonucleotides completely blocked Cl^- currents evoked by 10 nM LPA (Figs. 3*B* and 4*B*).

Pertussis Toxin Treatment of Oocytes Overexpressing xlp_{A1}-1 or xlp_{A1}-2—In *Xenopus* oocytes, endogenous LPA-evoked Cl^- currents have been documented to be unaffected by preincubation with PTX, consistent with the involvement of G_q pathways (28). Thus, we further examined whether XLP_{A1} might produce PTX-insensitive Cl^- currents. xlp_{A1}-*1*-injected oocytes were preincubated with PTX and electrophysiologically examined. As shown in Figs. 3*C* and 4*C*, PTX did not significantly affect LPA-evoked Cl^- currents. This observation demonstrated the involvement of PTX-insensitive G proteins in XLP_{A1}-mediated responses in *Xenopus* oocytes.

Heterologous Expression of XLP_{A1}-1 or -2 in Mammalian Cells—To examine additional signaling properties of XLP_{A1}-1 and -2 as compared with the known properties of mammalian LP_{A1}, each was expressed in B103 rat neuroblastoma cells (19) by infection with receptor-expressing recombinant retrovirus. The B103 cell line was chosen because it does not express any known LPA receptors and lacks endogenous responses to LPA, but does express appropriate α subunits of heterotrimeric G proteins ($G_{i/o}$, G_q, and $G_{12/13}$ subtypes) that can couple with LPA receptors (5, 13). Retroviral infection was used to introduce receptors into B103 cells to permit high efficiency expression and low cytotoxicity compared with conventional transfection methods (13).

To ascertain whether XLP_{A1}-1 or -2 was expressed in infected B103 cells, immunohistochemistry was used to identify

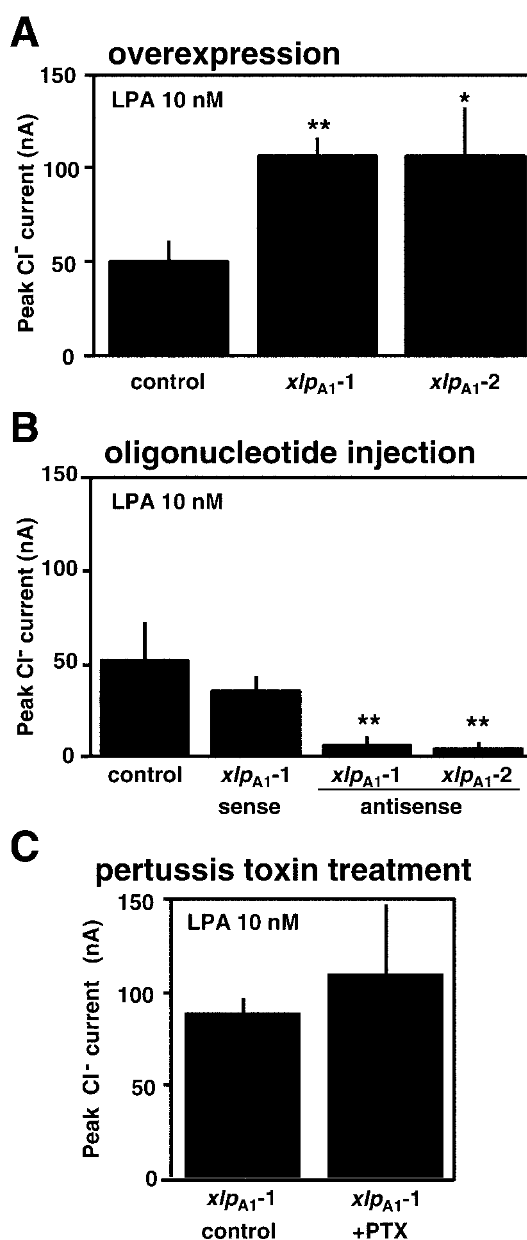

FIG. 4. **Statistical analyses of LPA-induced Cl⁻ currents in oocytes.** *A*, overexpression of XLP_{A1}-1 or XLP_{A1}-2. *B*, oligonucleotide injection. *C*, effect of PTX treatment. Data are the means ± S.E. (n = 6–9). **, $p < 0.01$; *, $p < 0.05$ as compared with controls.

the epitope (FLAG)-tagged receptors. FLAG-tagged protein was detected as punctate labeling in both soma and neurites (Fig. 5, *E, H*, and *K*). Consistent with retroviral mediated protein expression (see "Experimental Procedures" and Ref. 13), EGFP protein was also strongly and ubiquitously expressed in all receptor-expressing cells (Fig. 5, *A, D, G*, and *J*). Because the cells expressing EGFP proteins completely overlapped the cells expressing FLAG proteins (Fig. 5 and Ref. 13), EGFP fluorescence was used to monitor the infection efficiency before all functional assays. Typical infection percentages approximated 70–90%.

Mouse LP_{A1} mediates LPA-induced cytoskeletal reorganization including cell rounding in B103 cells and stress fiber formation in RH7777 rat hepatoma cells (5, 6, 13). To examine whether XLP_{A1}-1 and XLP_{A1}-2 also mediate cell rounding, B103 cells were infected, treated with various concentrations of LPA for 15 min, double-immunostained against EGFP and FLAG-tagged proteins, and observed by fluorescence micros-

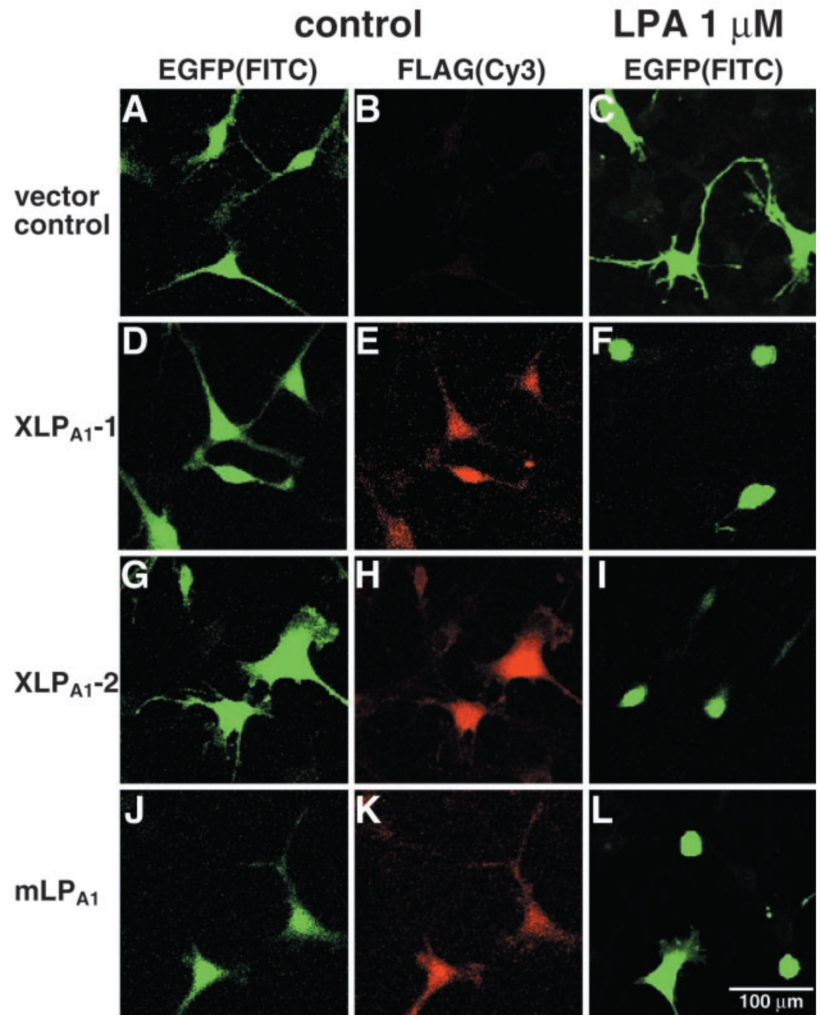

FIG. 5. **Confocal microscopy of cells heterologously expressing XLP_{A1}-1 and XLP_{A1}-2.** Confocal laser-scanning microscopy of B103 cells infected with control retrovirus (vector control) (*A–C*), XLP_{A1}-1-expressing retrovirus (*D–F*), XLP_{A1}-2-expressing retrovirus (*G–I*), and mouse LP_{A1}-expressing retrovirus (*J–L*). Cells were treated with 1 μM LPA for 15 min (*C*, *F*, *I*, and *L*). After fixation, cells were double-immunostained for EGFP (fluorescein isothiocyanate (*green*) in *A, C, D, F, G, I, J*, and *L*) and FLAG epitope (Cy3 (*red*) in *B, E, H*, and *K*). Cells expressing LP_{A1}s (XLP_{A1}-1, XLP_{A1}-2, and mouse LP_{A1}) showed a marked increase in cell rounding in response to LPA as compared with controls.

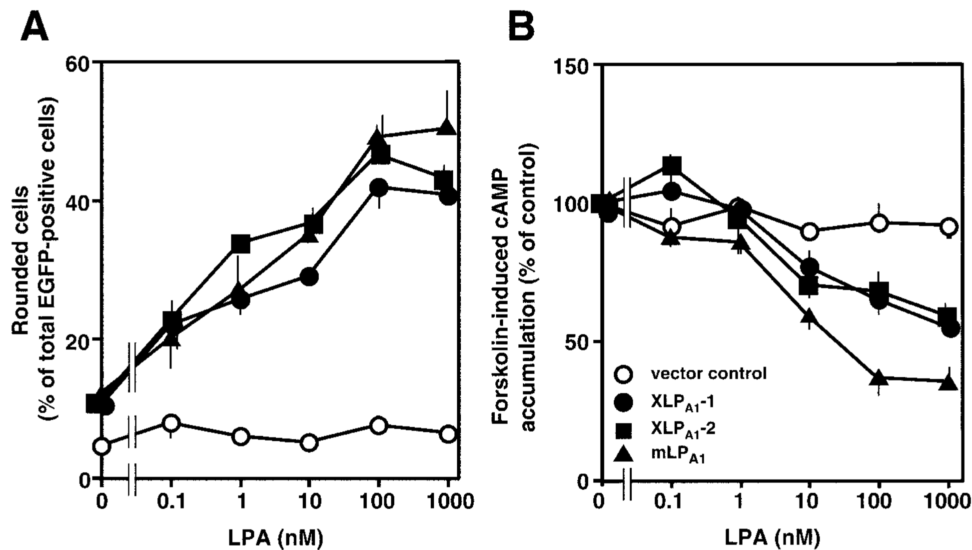

FIG. 6. **XLP_{A1}-1 and XLP_{A1}-2 mediate cellular LPA responses in B103 cells.** *A*, LPA concentration-dependent cell rounding in cells heterologously expressing XLP_{A1}-1 or XLP_{A1}-2, as compared with positive (mouse LP_{A1}-infected cells) and negative (vector-only infected cells) controls. Infected B103 cells were treated with LPA for 15 min, fixed, and immunostained. The number of rounded cells was expressed as a percentage of EGFP-positive cells (>200 cells/well). Data are the means ± S.E. (n = 4). *B*, LPA concentration-dependent inhibition of cAMP accumulation by XLP_{A1}-1 and XLP_{A1}-2 expression. Infected B103 cells were incubated with forskolin (1 μM) and LPA for 15 min. Forskolin-induced cAMP accumulation (750.1–1183.6 fmol/well) was expressed as 100%. Data are the means ± S.E. (n = 3).

copy. Without treatment, B103 cells had neurites protruding from the cell body (Fig. 5, *A, D, G*, and *J*), and after LPA stimulation, cells infected with control virus did not change their shapes (Figs. 5*C* and 6*A*). In contrast, B103 cells expressing XLP_{A1}-1 or XLP_{A1}-2 and exposed to LPA resulted in an increase in rounded cells with retracted neurites (Figs. 5, *F* and

I, and 6*A*). The maximal effects and EC_{50} values (~10 nM) for LPA were comparable with those observed for cells expressing mouse LP_{A1} (Figs. 5*L* and 6*A*). LPA-induced cell rounding in cells expressing XLP_{A1}-1 or XLP_{A1}-2 was completely inhibited by pretreatment with a Rho kinase inhibitor, Y-27632 (29), but not with PTX pretreatment (data not shown).

As with B103 cells, RH 7777 cells neither express any known LPA receptors nor show endogenous response to LPA (5). However, heterologous expression of mouse LP_{A1} in these cells produces LPA-dependent stress fiber formation (5). Heterologous expression of XLP_{A1}-1 or XLP_{A1}-2 increased the percentage of cells with stress fibers following LPA stimulation (1.9% in control cells, 29.1% in XLP_{A1}-1-expressing cells, and 32.0% in XLP_{A1}-2-expressing cells). This effect was comparable in extent and completely indistinguishable from the stress fibers produced in previous studies of mouse LP_{A1}-expressing cells (5).

Mouse LP_{A1} mediates LPA-induced inhibition of adenylyl cyclase in B103 cells (13), TR immortalized neuroblast cells (6), and HTC4 hepatoma cells (11). Infected B103 cells were incubated with forskolin (1 μM) in the absence or presence of various concentrations of LPA for 15 min. Intracellular cAMP content was measured by enzyme immunoassay. Forskolin-induced cAMP accumulation in B103 cells expressing XLP_{A1}-1 or XLP_{A1}-2 was inhibited by LPA treatment (Fig. 6*B*). Maximal inhibition in both was ~32% and was smaller than that observed in cells expressing mouse LP_{A1} (~63%). However, the EC_{50} values for inhibition were comparable among XLP_{A1}-1, XLP_{A1}-2, and mouse LP_{A1} (~10 nM). This inhibition was completely blocked by PTX pretreatment (data not shown).

DISCUSSION

In this study, we identified and characterized two novel *Xenopus* GPCRs, XLP_{A1}-1 and XLP_{A1}-2. Based on nucleotide and amino acid sequence similarities, endogenous expression in *Xenopus* tissues, and their function in both oocytes and mammalian cells, XLP_{A1}-1 and XLP_{A1}-2 are functional *Xenopus* homologs of the mammalian high affinity LPA receptor LP_{A1}.

A comparison of nucleic acid sequences, including divergent untranslated regions, and predicted amino acid sequences of *xlp*$_{A1}$*-1* and *xlp*$_{A1}$*-2* indicates that they are derived from two distinct genes rather than generated by alternative splicing of a single gene. The existence of multiple genes for a given function is not uncommon for *Xenopus laevis* (20, 30–33), in part reflecting the occurrence of genome duplication in *Xenopus* that results in a tetraploid (allotetraploid) genome (34).

xlp$_{A1}$*-1* and/or *-2* mRNA was expressed at high levels in oocytes and at lower levels in brain and spinal cord among the various *Xenopus* tissues examined. Alignment of amino acid sequences revealed that both XLP_{A1}-1 and XLP_{A1}-2 were highly similar to mammalian LP_{A1} (~89% identity) (3, 4, 35). By contrast, no signal was detected after Northern blot hybridization with mouse LP_{A2} or LP_{A3} probes under conditions allowing detection of XLP_{A1}-1 and XLP_{A1}-2 using mouse LP_{A1} (data not shown). This result suggests that *Xenopus* homologs of mammalian LP_{A2} or LP_{A3} do not exist in the *Xenopus* tissues examined or, at least, not those with the same degree of similarity as between mouse and *Xenopus* LP_{A1}.

In *Xenopus* oocytes, endogenous LPA-evoked Cl^- currents have been reported to be mediated through the activation of G_q and phospholipase C, based on studies using pharmacological or antisense oligonucleotide approaches (28, 36). In studies of mammalian LP_{A1}, it was recently reported that LPA stimulates phospholipase C through G_q pathways (13). Combined with data from the present study including PTX-insensitive augmentation of LPA-evoked Cl^- currents by *xlp*$_{A1}$, we conclude that endogenous LPA responses probably are mediated by G_q activation via XLP_{A1}s.

In addition to G_q activation, both XLP_{A1}s stimulate Rho (perhaps through $G_{12/13}$) and G_i pathways, resulting in cell rounding and stress fiber formation, and inhibition of cAMP accumulation, respectively. All responses are similar to those observed directly in previous studies of mammalian LP_{A1} (5, 13). The EC_{50} of LPA for cytoskeletal changes, as well as adenylyl cyclase inhibition in cells expressing XLP_{A1}, is below 10 nM, which is comparable with that of mouse LP_{A1} (5, 13). These results strongly support the identification of XLP_{A1}-1 and -2 as LPA receptors in *Xenopus*.

Xenopus oocytes appear to express both high and low affinity receptors for LPA based on electrophysiological studies of LPA-dependent Cl^- currents (25, 37, 38). Guo *et al.* (15) have reported a high affinity site for LPA with an EC_{50} of 12 nM and a low affinity site of 1 μM. Here, we observed that overexpression of XLP_{A1}-1 or -2 potentiated the Cl^- currents evoked by application of low concentrations (3–10 nM) of LPA. Injection of antisense oligonucleotide designed to inhibit expression of endogenous XLP_{A1}-1 and -2 completely inhibited the Cl^- currents evoked by the application of 10 nM LPA. Combined with the data from heterologous expression, we conclude that XLP_{A1}-1 and XLP_{A1}-2 are high affinity LPA receptors in *Xenopus* oocytes.

It should be noted that a different gene, *PSP24*, was previously reported to encode *Xenopus* high affinity LPA receptor, based on the potentiation of LPA-evoked Cl^- currents in oocytes following its overexpression (15). *Xenopus* PSP24 is dissimilar to mammalian LPA receptors, having less than 20% amino acid sequence identity with mammalian LP_{A1} (4). Moreover, heterologous expression of *Xenopus* PSP24 in yeast and of mammalian orthologs of PSP24 in mammalian cells does not mediate LPA responses (7, 16). Our data did not directly address the role of PSP24, because experimental results focused on XLP_{A1}-1 and XLP_{A1}-2 were sufficient to account for measured LPA responses in *Xenopus* oocytes. It is possible that an indirect relationship exists between XLP_{A1}-1 and XLP_{A1}-2 and PSP24, although we note the existence of technical difficulties in examining altered gene expression or post-translational changes in single, injected, and electrophysiologically characterized oocytes. The precise mechanism for LPA-evoked effects related to PSP24 in oocytes remains for future work, along with the question of what receptor mechanisms mediate the low affinity LPA interactions, which were not addressed in this study.

In summary, we have identified and characterized two high affinity *Xenopus* LPA receptors, both of which are similar in structure and function to mammalian LP_{A1} receptors. The existence of XLP_{A1}s provides a more evolutionarily frugal mechanism for LPA signaling that appears to be conserved from *Xenopus* through humans.

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