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Molecular cloning of a high-affinity receptor for the growth factor-like lipid mediator lysophosphatidic acid from *Xenopus* oocytes

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ABSTRACT **Lysophosphatidic acid (1-acyl-2-lyso-*sn*-glycero-3-phosphate, LPA) is a multifunctional lipid mediator found in a variety of organisms that span the phylogenetic tree from humans to plants. Although its physiological function is not clearly understood, LPA is a potent regulator of mammalian cell proliferation; it is one of the major mitogens found in blood serum. In *Xenopus laevis* oocytes, LPA elicits oscillatory Cl^- currents. This current, like other effects of LPA, is consistent with a plasma membrane receptor-mediated activation of G protein-linked signal transduction pathways. Herein we report the identification of a complementary DNA from *Xenopus* that encodes a functional high-affinity LPA receptor. The predicted structure of this protein of 372 amino acids contains features common to members of the seven transmembrane receptor superfamily with a predicted extracellular amino and intracellular carboxyl terminus. An antisense oligonucleotide derived from the first 5–11 predicted amino acids, selectively inhibited the expression of the endogenous high-affinity LPA receptors in *Xenopus* oocytes, whereas the same oligonucleotide did not affect the low-affinity LPA receptor. Expression of the full-length cRNA in oocytes led to an increase in maximal Cl^- current due to increased expression of the high-affinity LPA receptor, but activation of the low-affinity receptor was, again, unaffected. Oocytes expressing cRNA prepared from this clone showed no response to other lipid mediators including prostaglandins, leukotrienes, sphingosine 1-phosphate, sphingosylphosphorylcholine, and platelet-activating factor, suggesting that the receptor is highly selective for LPA.**

1-Acyl-2-lyso-*sn*-glycero-3-phosphate (LPA) is nature's simplest glycerophospholipid, which elicits growth factor-like effects on cell proliferation (1, 2) and cell migration (3); and, because it is generated during blood clotting (4), it has been suggested that LPA plays a role in wound healing and regeneration (5). Recently, evidence has been presented suggesting that LPA can suppress apoptosis *in vitro* as well as in ischemic organs such as heart and liver (6). There is considerable circumstantial evidence supporting plasma membrane receptors for LPA (7), and LPA-binding proteins have been reported in mammalian tissues (8) and labeled using a photoaffinity crosslinker derivative (9). The ubiquitous presence of the response elicited by LPA in almost every cell line tested, combined with the amphiphilic character of LPA that makes radioligand binding assays extremely difficult, has presented considerable difficulties in the molecular cloning of its receptors. Herein we report the molecular cloning of a complementary DNA that encodes a functional LPA receptor in *Xenopus* oocytes. The putative LPA receptor has features common to the family of seven transmembrane spanning receptors and functions as a high-affinity LPA receptor, whereas an antisense oligonucleotide derived from it selectively blocks the expression of the high affinity LPA receptor in oocytes.

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MATERIALS AND METHODS

Oocytes that were freed of the surrounding follicular and epithelial cell layers by collagenase treatment and manual peeling were used for extraction of poly(A)$^+$ mRNA by methods described previously (7). The first-strand cDNA was synthesized using a 3′ rapid amplification of cDNA ends (RACE) kit from Life Technologies (Gaithersburg, MD). Polymerase chain reactions (PCRs) were carried out using *Taq* I polymerase (Stratagene) with dNTPs (Boehringer Mannheim) in an OmniGene thermocycler (Hybaid, Middlesex, U.K.). Amplification products were excised from agarose gels and cloned into the T-tail vector from Novagen. DNA sequencing was performed by the dideoxynucleotide chain-termination method with a BST kit (Bio-Rad). A directional oocyte cDNA library was prepared by the Superscript plasmid system (Life Technologies) using the *Not*I and *Sal*I restriction sites of the pSPORT1 vector. Colony hybridization was assayed with the Zeta-Probe GT-blotting system (Bio-Rad). The oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems model 394 DNA/RNA synthesizer, purified by polyacrylamide gel electrophoresis, and desalted on Sep-Pak C_{18} cartridges (Waters). Full-length cRNA was synthesized using T7 polymerase with the mCAP kit (Stratagene), whereas antisense cRNA was prepared by using Sp6 polymerase. The platelet-activating factor (PAF) receptor cDNA was a gift from Takao Shimizu (Tokyo University). Expression of cRNA and culture of oocytes was done using previously established methods (10). Electrophysiological recording from oocytes was carried out as described (7).

RESULTS AND DISCUSSION

The cloning strategy pursued herein originated from the observation that microinjection of antisense cRNA derived from the sequence of the platelet-activating factor (1-alkyl-2-acetyl-*sn*-glycerophosphocholine, PAF) receptor into *Xenopus* oocytes inhibited the endogenous LPA response, thus suggesting that homology existed between these G protein-coupled receptors (Fig. 1*a*). Because of the similarities in the cellular

Abbreviations: LPA, lysophosphatidic acid (1-acyl-2-lyso-*sn*-glycero-3-phosphate); cLPA, cyclic LPA (1-acyl-*sn*-glycero-2,3-cyclic phosphate); PAF, platelet-activating factor; PAFR, platelet-activating factor receptor.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U76385).

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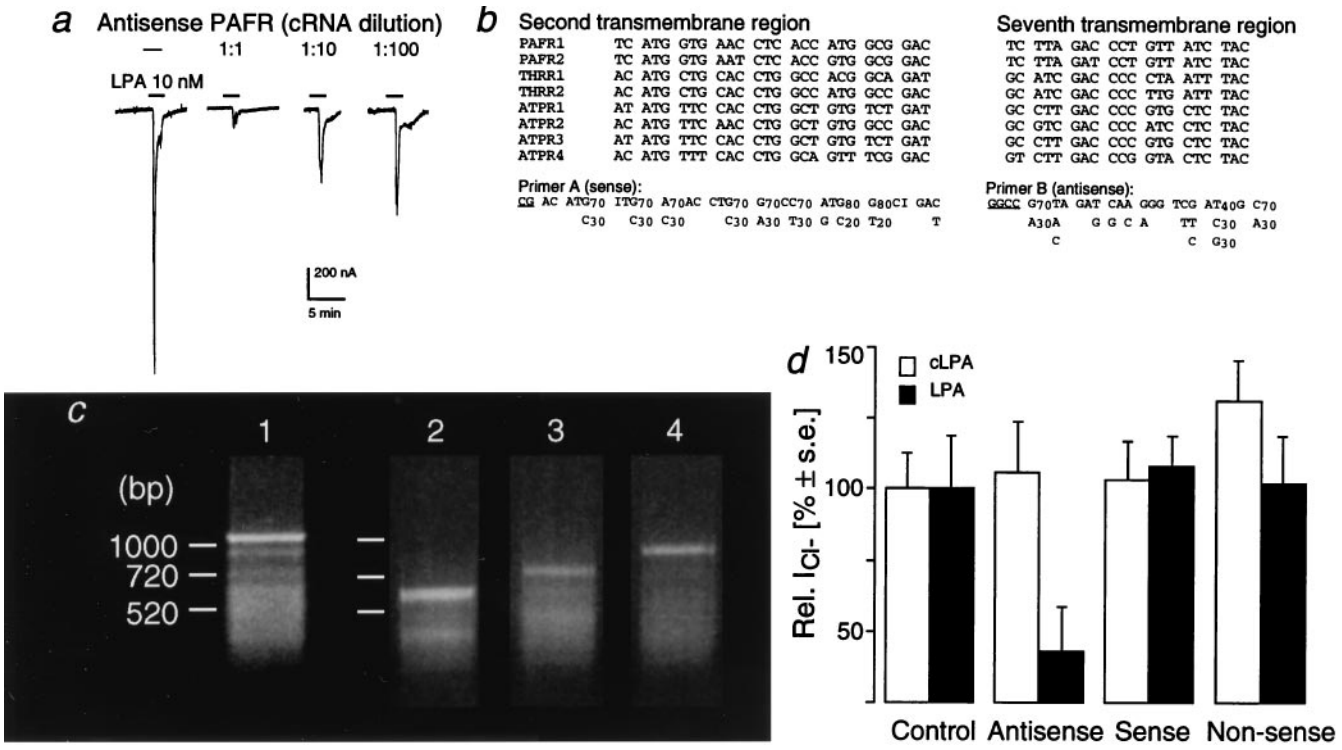

FIG. 1. (*a*) Antisense PAFR cRNA inhibits the endogenous LPA response in *Xenopus* oocytes. Oocytes were injected with increasing dilutions of a stock solution containing full-length PAFR antisense cRNA at 2 ng/nl. Responses to LPA were recorded up to 6 days later using a standard two-electrode voltage-clamp configuration with the membrane potential held at −60 mV. LPA was applied via superfusion at a concentration of 10 nM. Similar conditions were used throughout the experiments presented. Injection of PAFR antisense cRNA inhibited the LPA response in a dose-dependent fashion. (*b*) Design of degenerate oligonucleotides for PCR. Nucleotide sequence alignment of receptors for PAF (PAFR1, human; PAFR2, rat), thrombin (THR) (THR1, human; THR2, mouse), and ATP (ATPR1-4, human, chicken, turkey, and mouse, respectively) in the second and seventh transmembrane regions. Subscript numbers next to nucleotides in primers A and B represent their relative proportions used during synthesis. (*c*) PCR products isolated from the oocyte. Lanes of 1% agarose gels show the major amplification products obtained from the first PCR with primer A and the universal primer of the 3′-RACE kit (lane 1). Products of the nested PCR with primers A and B using the >1.2-kb template from the first reaction were cloned into the T-tail vector and three clones, representative of the three major PCR products, are shown in lanes 2–4 with different insert sizes (600–800 bp). (*d*) An 18-mer oligonucleotide (≈0.3 fmol per oocyte) derived from clone 71 selectively inhibits the endogenous LPA response, whereas it does not effect the cLPA response. The bar graph shows normalized mean currents to the low-affinity receptor-selective agonist cLPA (open bars, 1 μM) and the nonselective agonist LPA (solid bars, 10 nM). Injection of sense and random-sequence oligonucleotides did not affect the responses significantly.

responses elicited by receptors for PAF, thrombin, and ATP and the coupling of these receptors to the G_q, $G_{12/13}$, and G_i subtypes of heterotrimeric G proteins, a search for nucleotide sequence motifs common to this group was carried out. Alignment of these sequences identified two conserved regions in the second and seventh putative transmembrane segments (Fig. 1*b*). These motifs were used to design degenerate oligonucleotide primers for PCR (Fig. 1*b*). *Xenopus* oocytes do not express endogenous receptors to PAF, thrombin, or ATP, whereas there are at least two pharmacologically different subtypes of LPA receptors present in the oocyte (7, 11, 12). These two LPA receptor subtypes are distinguished by the naturally occurring lipid mediator cyclic-LPA (1-acyl-*sn*-glycero-2,3-cyclic phosphate, cLPA), which selectively activates the low-affinity but not the high-affinity receptor (7). Hence, the oocyte was chosen for the source of template mRNA for reverse transcription-coupled PCR. The first PCR amplification was performed using degenerate primer A (Fig. 1*b*) and a universal primer with the sequence of 5′-GGCCACGCGTCGACTAGTAC-3′. The >1.2-kb products of this amplification (Fig. 1*c*) were excised from the agarose gel and used in a reamplification reaction with the nested application of degenerate primers A and B (Fig. 1*b*). The second PCR resulted in the amplification of three major products with sizes greater than 500 bp (Fig. 1*c*). One of the products, ≈800 bp long, agreed with the predicted size of a hypothetical G-protein-coupled receptor from the second to the seventh transmembrane region. This product was cloned into the T-tail vector and a total of 19 clones were selected for sequencing. One of these, designated clone 71, contained a novel sequence, to our knowledge, not present in the GenBank database and had features resembling those of a G-protein-coupled receptor. This clone, with a 798-bp insert, constituted the N-terminal portion of a novel protein including a 106-bp sequence upstream from the initiation codon. Starting at base 57, upstream from the ATG initiation codon of the predicted open reading frame, an in-frame signal peptide was detected that is often found in genes encoding seven transmembrane spanning receptors (13).

To examine the relationship of clone 71 to the putative LPA receptor, an 18-mer antisense oligonucleotide with the sequence of 5′-ATTGTCTAGGGCAGTATT-3′, complementary to the putative N-terminal 5–11 amino acids, was synthesized and injected into *Xenopus* oocytes. A sense- and a random-sequence oligonucleotide, containing the same relative percentages of the four nucleotides, served as controls. Oscillatory Cl^- currents elicited by LPA and cLPA measured with standard two-electrode voltage-clamp recording in the three groups of oligonucleotide-injected oocytes (Fig. 1*d*) showed no statistically significant differences in the response to cLPA nor were there any differences seen in the oocytes injected with distilled water. In contrast, the LPA response was substantially diminished, by 68 ± 12%, in the group injected with as little as 0.3 fmol of the antisense oligonucleotide. Oocytes injected with sense and nonsense oligonucleotides

showed LPA responses similar to those of sham-injected controls (Fig. 1*d*). These results suggested that the sequence derived from the N-terminal 5–11 amino acids of clone 71 was homologous with that of the type 1 (high-affinity) LPA receptor.

The full coding sequence of this novel gene was identified using colony hybridization screening in a cDNA library prepared from oocyte poly(A)$^+$ mRNA. In a library of $\approx 10^6$ colonies, high-stringency library screening for homologues of clone 71 yielded 69 positive colonies, 5 of which were identical and contained the full coding sequence of a novel gene. The entire sequence of 1 of the 5 clones, designated PSP24, is shown in Fig. 2. The 1119-bp open reading frame derived from the cDNA sequence encodes a protein of 372 amino acids with a calculated molecular weight of 42,263 and an estimated pI of 10.48. Analysis of the hydropathy profile of the amino acid sequence predicts seven putative membrane-spanning regions, characteristic of G-protein-coupled receptors. Computer-assisted sequence analysis predicts a G-protein-binding site between the putative transmembrane regions 1 and 2 on the intracellular side of the protein. The N terminus is predicted to be extracellular and contains two potential N-glycosylation sites at Asn-4 and Asn-15. A 58-amino acid long tail at the C terminus carries two consensus phosphorylation sites for protein kinase C (PKC). In addition, there are two more PKC sites predicted between the fifth and sixth transmembrane segments. These sites might play a role in the PKC-mediated desensitization of the LPA receptor (14). Sequence analysis identified a number of other potential phosphorylation sites, including Thr-231 (which is a target for the cAMP-activated kinases), the Ca^{2+}/calmodulin-dependent type II kinases, and the casein kinases. Thr-361 is found within a consensus recognition site for phosphorylation by the cAMP-activated and Ca^{2+}/calmodulin-dependent type II kinases, whereas Lys-339 is a potential methylation site. The functional consequences of these potential protein modifications remain to be elucidated, particularly because LPA-induced cellular responses involve both cAMP and Ca^{2+}-mediated signal transduction mechanisms. There are pairs of cysteine residues in the N-terminal region, in the intracellular segment between the fifth and sixth predicted transmembrane domains and also in the C-terminal tail, creating the potential for the formation of disulfide bridges. A search of the GenBank data base for genes with nucleotide sequence similarity to that of clone PSP24 revealed a number of G-protein-coupled receptors. In addition to the PAF, thrombin, and purinergic receptors used in the primer design leading to the isolation of clone PSP24, the neuropeptide Y, β_1-adrenergic receptor, histamine type 2 receptor, α_2-adrenergic receptor, and the bombesin receptor showed the highest degree of homology, particularly in the 5′ region of the sequence. Alignment of the putative amino acid sequence of clone PSP24 with that of members of the PAF receptor (PAFR) family identified 64 identical and 90 conserved residues, establishing an overall homology of 41% (Fig. 2).

cRNA transcribed *in vitro* from clone PSP24 was expressed in oocytes. Oocytes injected with PSP24 cRNA showed no oscillatory Cl^- current responses to PAF, ATP, thrombin, or a variety of other lipid mediators, including various prostaglandins, leukotrienes, sphingosine 1-phosphate, and sphingosylphosphorylcholine (Fig. 3*a*). In contrast, responses to LPA were increased 1.5- to 10-fold over those of water-injected control oocytes. The dose–response relationship of the LPA-elicited currents in oocytes injected with 0.5 ng of PSP24 cRNA showed a much steeper curve compared with that of sham-injected control oocytes (Fig. 3*b*). Mathematical analysis of the LPA dose–response relationship in the cRNA-injected oocytes and in sham-injected oocytes, using a previously established model (7, 12) and assuming the simultaneous expression of at least two independent LPA receptors (7), permitted nonlinear fitting of the data with the sums of two Langmuir isotherms using the Levenberg–Marquard algorithm. This procedure identified at least two, a high- and a low-affinity LPA receptor in both the control and PSP24 cRNA-expressing oocytes. The apparent EC_{50} values for the receptors changed slightly, revealing 7.8 ± 1.5 vs. 7.4 ± 0.8 nM for the high-affinity and 540 ± 200 vs. 820 ± 530 nM for the low-affinity receptors in sham-injected vs. PSP24 cRNA-injected oocytes, respectively. In contrast, an overall 2-fold increase in the maximal LPA-activated Cl^- current was detected. In a representative experiment, the increase in the maximum current from 920 ± 100 nA (n = 4 oocytes) to 1890 ± 130 (n = 5) was caused by activation of the high-affinity site, whereas the maximum current contributed by the low-affinity site remained unchanged (1080 ± 90 vs. 1120 ± 180 nA). When a linear relationship between receptor occupancy and response size is assumed [described by the equation $I = c_1R_{1L} + c_2R_{2L}$, where I is the current, c_1 and c_2 are coupling-efficiency constants for the two receptors, and R_{1L} and R_{2L} are concentrations of receptor–ligand complexes (15)] these data indicate a minimum 2-fold increase in the number of receptor sites expressed in the oocyte after the injection of PSP24 cRNA in this particular experiment. Analysis of the same data, shown in Fig. 3*b*, was also carried out using a more general model proposed by Kenakin (16), which operates with a logistic transducer function for the activation of two receptors by the same ligand. Calculations using the Kenakin model, $I/I_{max} = (R_{1L} + \mu R_{2L}) \times 1/(1 + \beta)$, where μ is a proportionality factor and β is defined as an efficiency factor for tissue processing of the stimulus (16), yielded essentially the same increase in the number of high-affinity receptors. In sham-injected oocytes, the calculated K_d for the high-affinity site was 12.7 ± 2.5 nM and for the low-affinity site was 1.0 ± 0.3 μM, whereas in oocytes injected with PSP24 cRNA, the K_d values were 8.1 ± 1.5 nM and 0.9 ± 0.4 μM, respectively. As a result of the expression of PSP24 cRNA, the calculated maximal current activated by the high-affinity site increased from 1130 ± 90 nA to 1970 ± 130 nA, whereas the current contributed by the low affinity receptor remained unchanged (880 ± 150 nA vs. 1060 ± 180 nA). Given the large size of the LPA response in the native oocyte, which can generate currents of several microamperes, an average 2-fold increase in the number of functional receptors is likely to represent a substantial increase in the synthesis and expression of the receptor protein. However, until an accurate measure of the number of receptors present in the oocyte plasma membrane is available, the estimation of the exact number of receptor sites remains elusive.

Injection of the antisense oligonucleotide derived from clone 71 not only diminished the endogenous LPA response but also abolished the cRNA-induced enhancement of the LPA response (Fig. 3*c*). However, when the injection of PSP24 cRNA was delayed ≈48 h after injection of the antisense oligonucleotide, the LPA response enlarged ≈1.5-fold over that of the sham-injected controls. This indicates that the inhibition of the endogenous LPA response by the antisense oligonucleotide can be overcome by expression of full-length PSP24 cRNA. The increase in the LPA response was dependent on the amount of cRNA injected into the oocyte (Fig. 3*d*). In contrast, oscillatory Cl^- currents elicited by the low-affinity receptor-selective ligand cLPA in oocytes injected with PSP24 cRNA showed no statistically significant differences compared with those of the sham-injected controls (Fig. 3*d*). This finding suggests that the inhibition of the LPA response by the full-length antisense cRNA from clone PSP24 was highly selective for the high-affinity receptor, leaving the low-affinity cLPA receptor-mediated response unaffected. Furthermore, the lack of inhibition of the cLPA response indicates that the common signal transduction machinery shared by the two receptors in the same oocyte was unaffected by the expression of full-length antisense PSP24 cRNA, duplicating observations gained with the 18-mer oligonucleotide derived from clone 71 (Fig. 1*d*). The lack of effect of full-length antisense PSP24

a

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                                                                                -106 CCGCTGCATCCGGCTGGAAGATCTTTC
-79   AAGATGTGGGAAGAAAGGATCAATGCATCCTGCACAGTGAAGGCATCGCGAGAAGAACCATGCTAACAGGCTTCTAACA
1     ATG GGC TGC AAT AAT ACT GCC CTA GAC AAT TGC ATG CTT CCC AAT CTC AGC ATT GCT ACT
1     M   G   C   N   N   T   A   L   D   N   C   M   L   P   N   L   S   I   A   T

61    GCT CCT CTA GAC TTG AGG TTT GCA TTT TCC ACC CCA CTC AGG ATG TTG TTG GCA ATA ATA
21    A   P   L   D   L   R   F   A   F   S   T   P   L   R   M   L   L   A   I   I

121   ATG ATA CTG ATG ATT GCT ATT GCT TTT TTG GGC AAT GCA ATA GTT TGC CTT ATT GTT TAT
41    M   I   L   M   I   I   A   I   A   F   L   G   N   A   I   V   C   L   I   V   Y

181   CAG AAG CCA GCC ATG CGT TCA GCA ATC AAT CTT CTC CTA GCA ACA CTG GCA TTC TCT GAC
61    Q   K   P   A   M   R   S   A   I   N   L   L   L   A   T   L   A   F   S   D

241   ATC ATG TTG TCC CTT TTC TGT ATG CCC TTT ACC GCA GTA ACA ATA ATA ACT GGG AGC TGG
81    I   M   L   S   L   F   C   M   P   F   T   A   V   T   I   I   T   G   S   W

301   CTC TTT GGA ACT CAG TTT TGC CAG ATA TCA GCC ATG CTG TAC TGG TTT TTT GTG TTA GAA
101   L   F   G   T   Q   F   C   Q   I   S   A   M   L   Y   W   F   F   V   L   E

361   GGC GTG GCC ATT CTA CTT ATC ATC AGC GTG GAT CGT TTC CTT ATT ATT GTA CAG AGG CAG
121   G   V   A   I   L   L   I   I   S   V   D   R   F   L   I   I   V   Q   R   Q

421   GAC AAA CTG AAC CCA CAT CGG GCC AAG ATC ATG ATT GCT GCT TCT TGG GTA CTG TCT TTT
141   D   K   L   N   P   H   R   A   K   I   M   I   A   A   S   W   V   L   S   F

481   TGT ATC TCT TTA CCG TCG GTG GTT GGG TGG ACG TTA GTG GAG GTG CCT ACA CGT GCT CCA
161   C   I   S   L   P   S   V   V   G   W   T   L   V   E   V   P   T   R   A   P

541   CAG TGT GTT CTC GGT TAT ACA GAA TTT TCT GCT GAC AGA GTT TAT GCA GTC ATG CTC ATA
181   Q   C   V   L   G   Y   T   E   F   S   A   D   R   V   Y   A   V   M   L   I

601   GTA GCA GTC TTC TTC ATT CCA TTC AGT GTA ATG TTG TAC TCG TAT TTG TGT ATC CTG AAC
201   V   A   V   F   F   I   P   F   S   V   M   L   Y   S   Y   L   C   I   L   N

661   ACC GTT AGA AGA AAT GCT GTC AGA ATT CAC ACT CAT GCC GAC AGC TTG TGT CTC AGC CAA
221   T*  V   R   R   N   A   V   R   I   H   T*  H   A   D   S   L   C   L   S   Q

721   GTA AGC AAA TTG GGA CTT ATG GGA CTT CAG AGG CCA CAC CAA ATG AAT GTG GAC ATG AGT
241   V   S   K   L   G   L   M   G   L   Q   R   P   H   Q   M   N   V   D   M   S*

781   TTC AAA ACC AGG GCC TTC ACC ACT ATT TTG ATT CTC TTC ATT GGG TTT TCA CTC TGC TGG
261   F   K   T   R   A   F   T   T   I   L   I   L   F   I   G   F   S   L   C   W

841   CTT CCT CAC TCA GTA TTC AGC TTA CTT TCA GTA TTC AGC AGG ACA TTT TAC TAC AGC TCT
281   L   P   H   S   V   F   S   L   L   S   V   F   S   R   T   F   Y   Y   S   S

901   TCA TTT TAC AGC ATC AGC ACG TGT ACC TTG TGG CTC ACT TAC CTG AAG TCT GTC TTC AAC
301   S   F   Y   S   I   S   T   C   T   L   W   L   T   Y   L   K   S   V   F   N

961 CCT GTT ATA TAT TGC TGG AGG ATC AAG AAG TTC CGT GAG GCC TGT CTG GAG TTC ATG CCC
321   P   V   I   Y   C   W   R   I   K   K   F   R   E   A   C   L   E   F   M   P

1021  AAA ACA TTT AAG ATC CTT CCA AAT GTA CGA GGA AGG ACA AGA CGG CGG ATT CGT CCA AGC
341   K   T*  F   K   I   L   P   N   V   R   G   R   T*  R   R   R   I   R   P   S

1081  ACA ATT TAT GTG TGT GGG GAA CAT CAG TCA GCT GTT TAA GAAACCTCTTCAAGGAAATTTGTAAAA
361   T*  I   Y   V   C   G   E   H   Q   S   A   V   ***
1147  CGAGCACAAACAGTGTGGTTTGCCATTTTAAAAAGGTTTACATTTTCATGTTTTTTTGGTTTTTAATAACTGATTATAA
1226  AGCACAGGTCTTATTAGGTCACGTTCTTGTTTTGTATTTCATAACAATGCACAGTTTTGGGATGATAATGGTAAGGTGT
1305  GTAGCAGTTTTTCTTTAAAGAAAAAAAACAAAAGAAAAA
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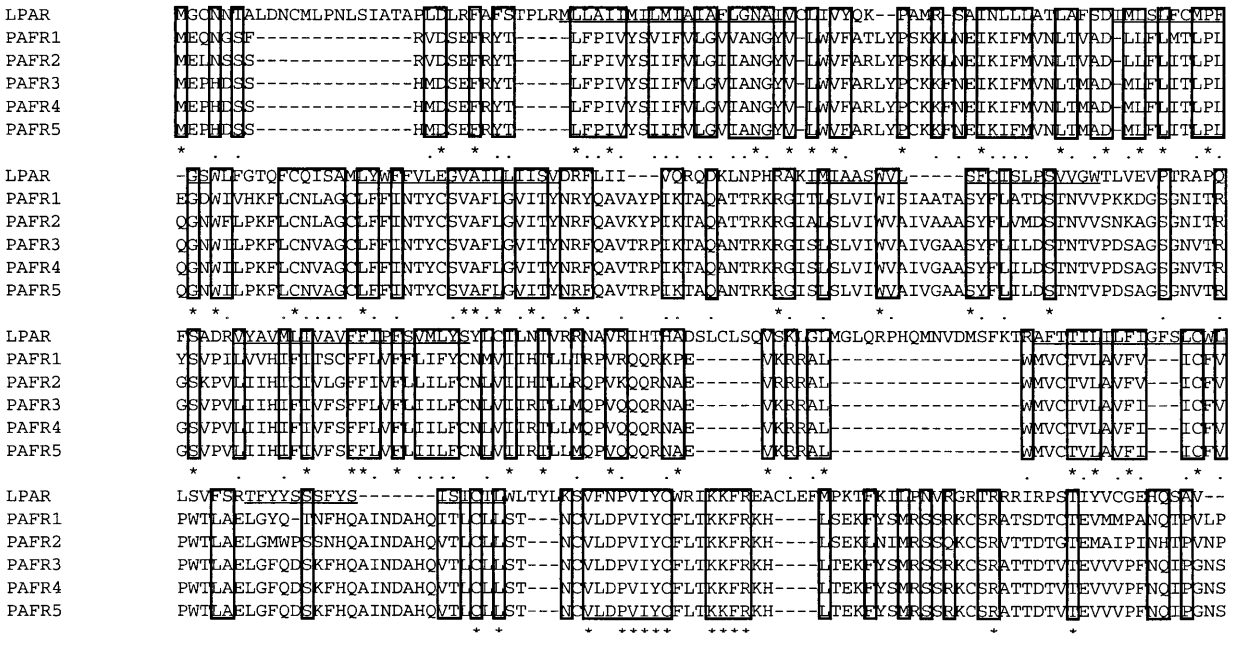
b



(*Fig. 2 legend appears on the opposite page.*)

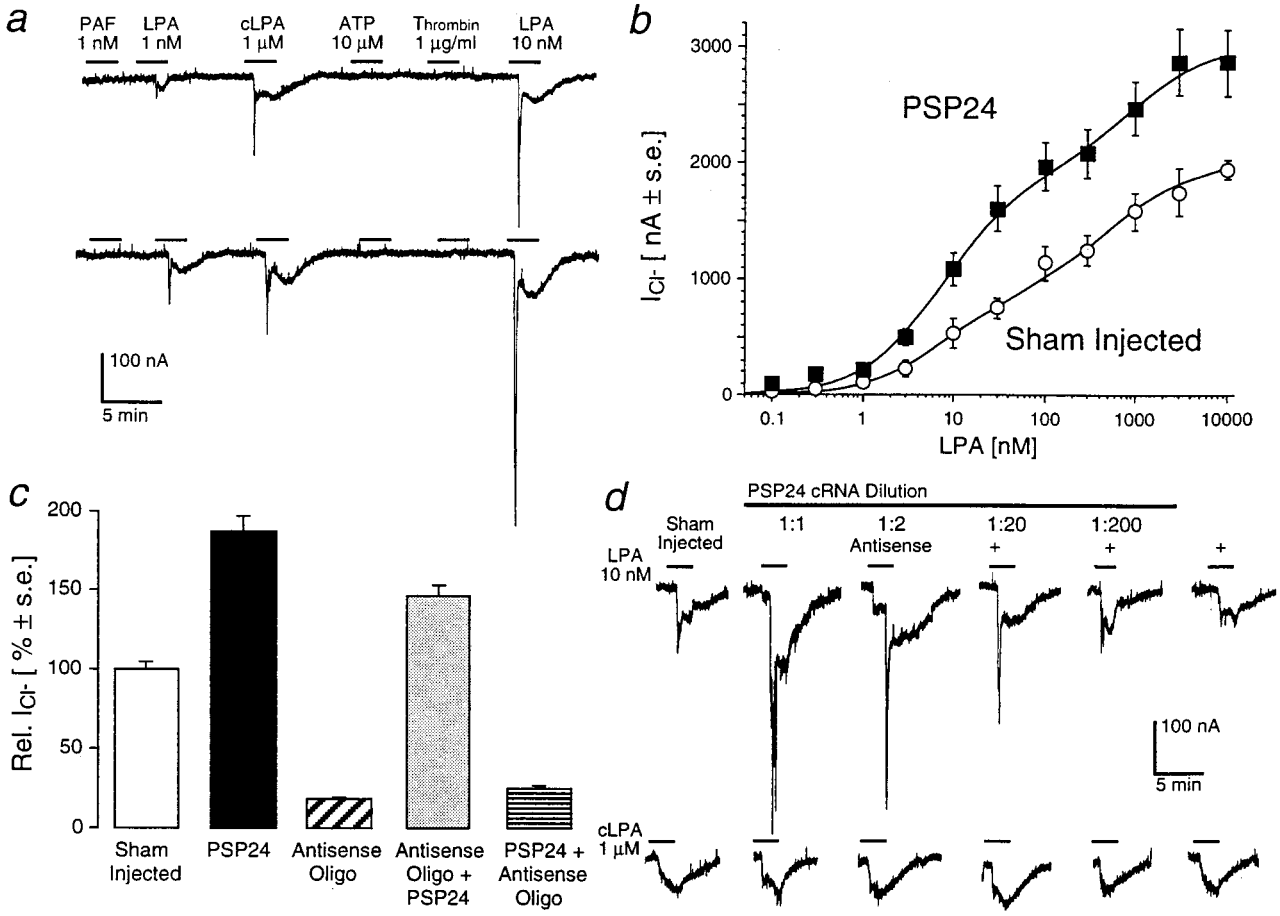


FIG. 3. (*a*) Representative responses to LPA, cLPA, and the other mediators used for primer design in control (upper trace) and PSP24 cRNA-injected (0.5 ng, lower trace) oocytes from the same frog. Notice that the cLPA response is practically identical in both cells, whereas the response to LPA is 2.5- to 3-fold higher (10 nM and 1 nM, respectively). None of the other mediators triggered an oscillatory response in either cell. (*b*) LPA dose–response relationship in sham-injected control (open circles, $n = 4$) and PSP24 cRNA-injected (solid squares, $n = 5$) oocytes. The responses in PSP24 cRNA-expressing oocytes exceeded those of the controls and showed an even more pronounced enhancement between 0.1 and 100 nM, contributing to the high-affinity receptor for LPA. (*c*) An antisense oligonucleotide designed from the sequence of amino acids 5–11 of clone PSP24 inhibits both the endogenous and the induced LPA response ($n = 6$ for all conditions). Injection of PSP24 cRNA at 0.5 ng per oocyte (solid bar) approximately doubled the response to 10 nM LPA in this experiment. Injection of ≈0.3 fmol (2.5 pg) of the 18-mer antisense oligonucleotide (hatched bar) reduced the endogenous response by 80%. To avoid the injection of double-stranded RNA that triggers the activation of nucleases, oocytes were injected a second time 2 days after the first injection with either PSP24 cRNA or the antisense oligonucleotide. A second injection of PSP24 cRNA (0.5 ng) into the oocytes (shaded bar) that were first injected with antisense oligonucleotide caused an ≈1.5-fold increase in the mean response over that of the distilled water-injected ("sham-injected") controls. A second injection of the antisense oligonucleotide into oocytes that were first injected with PSP24 cRNA inhibited the response 73% below the endogenous response. All bars represent the mean response (±SEM) for six oocytes. (*d*) The increase in the LPA response is selective and dependent on the amount of PSP24 cRNA present. In this experiment, oocytes were injected with a 1- to 200-fold dilution of a stock solution of PSP24 cRNA (1 μg/μl, 50 nl per oocyte). A constant amount (0.3 fmol) of antisense cRNA was mixed with and coinjected with increasing dilutions of sense cRNA. Responses to 10 nM LPA and 1 μM cLPA were measured 4–6 days after injection. Traces are representative of four independent determinations ($n = 4$). Note that the increase in the LPA response decreased with the decreasing amount of PSP24 cRNA injected, whereas the cLPA response was virtually unaffected in all groups. Injection of 0.3 fmol of antisense cRNA, alone, caused a 45% reduction in the endogenous LPA response, whereas the cLPA response was virtually unaltered.

cRNA on the cLPA response corroborates suggestions made in earlier reports (7, 12), based entirely on pharmacological evidence, that the low-affinity cLPA receptor was distinct from the type 1, high-affinity LPA receptor. Injection of full-length antisense PSP24 cRNA (Fig. 3*d*) or antisense oligonucleotide (Fig. 3*c*) not only attenuated the endogenous LPA response but also diminished the enhancement induced by sense PSP24 cRNA. Finally, the enhancement of the LPA response in oocytes injected with PSP24 cRNA increased with time, reaching its maximum between 2 and 3 days after microinjection of 0.5 ng of cRNA per oocyte. Simultaneous expression of sense cRNA encoding the type 2c serotonin and PAFRs, which also activate oscillatory Cl^- currents, had no effect on the expression of the endogenous LPA and cLPA response nor did

FIG. 2. (*a*) Nucleotide sequence of clone PSP24. Nucleotides underlined with a solid line represent part of the sequence encoding a putative signal peptide. Amino acid residues underlined indicate the predicted positions of the seven transmembrane regions. The dashed underline, in the amino acid sequence, marks the possible polyadenylylation site. Amino acids in boldface type and marked with asterisks represent possible phosphorylation sites, whereas those in italic type are potential N-glycosylation sites. The three asterisks mark the stop codon at the end of the open reading frame. (*b*) The putative amino acid sequence of the LPA receptor (LPAR) encoded by clone PSP24 shows sequence homology with different PAFRs (PAFR1, rat; PAFR2, guinea pig; PAFR3, human leukocyte; PAFR4, human heart; PAFR5, human granulocyte). Blocks drawn around the sequence mark conserved motifs. Asterisks under the sequence mark identical residues, whereas dots indicate conserved residues yielding a 17% sequence identity and an overall homology of 41% between these receptors.

it affect the increased LPA responsiveness induced by PSP24 cRNA (data not shown).

Northern blot analysis has revealed the ubiquitous presence of the receptor mRNA (data not shown). Identification of clone PSP24 as a gene encoding a putative LPA receptor is certainly only the first step toward a better understanding of the role of these fairly ubiquitous receptors. There is already ample evidence for the existence of multiple types of LPA-activated receptors that are often expressed simultaneously in the same cell (7, 12). Therefore, it is likely that more genes encoding receptors activated by LPA will be discovered in the near future, not only advancing our understanding of these lipid mediators but also providing grounds for their therapeutic application as alternatives to polypeptide growth factors.

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