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Comparative analysis of three murine G-protein coupled receptors activated by sphingosine-1-phosphate

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Abstract

The cloning and analysis of the first identified lysophosphatidic acid (LPA) receptor gene, lp_{A1} (also referred to as *vzg-1* or *edg-2*), led us to identify homologous murine genes that might also encode receptors for related lysophospholipid ligands. Three murine genomic clones (designated lp_{B1}, lp_{B2}, and lp_{B3}) were isolated, corresponding to human/rat *Edg-1*, rat H218/AGR16, and human edg-3, respectively. Based on the amino acid similarities of their predicted proteins (44–52% identical), the three lp_B genes could be grouped into a separate G-protein coupled receptor subfamily, distinct from that containing the LPA receptor genes lp_{A1} and lp_{A2}. Unlike lp_{A1} and lp_{A2}, which contain multiple coding exons, all lp_B members contained a single coding exon. Heterologous expression of individual lp_B members in a hepatoma cell line (RH7777), followed by ^{35}S-GTPγS incorporation assays demonstrated that each of the three LP_B receptors conferred sphingosine-1-phosphate-dependent, but not lysophosphatidic acid-dependent, G-protein activation. Northern blot and in situ hybridization analyses revealed overlapping as well as distinct expression patterns in both embryonic and adult tissues. This comparative characterization of multiple sphingosine-1-phosphate receptor genes and their spatiotemporal expression patterns will aid in understanding the biological roles of this enlarging lysophospholipid receptor family.

Keywords: edg; GPCR; Lysophosphatidic acid; Lysophospholipid[1]

1. Introduction

Lysophosphatidic acid (LPA; 1-acyl-2-*sn*-glycerol-3-phosphate) and sphingosine-1-phosphate (S1P; 1-phospho-2-amino-4-*trans*-octadecene-1,3-diol) are bioactive lysophospholipids that can stimulate a large range of responses in various cell types. These responses include acute neurite retraction, actin stress-fiber formation, cell proliferation, Ca^{++} and Cl^- conductance changes, smooth muscle contraction, and platelet activation (Durieux, 1995; Moolenaar et al., 1997; Spiegel et al., 1998). Though some S1P effects appear to be mediated intracellularly, it is now evident that both LPA and S1P signal through specific G-protein coupled receptors (GPCRs; Hecht et al., 1996; Fukushima et al., 1998; Lee et al., 1998b; Zondag et al., 1998). The first such GPCR identified, *ventricular zone gene-1* (*vzg-1*, now referred to as lp_{A1}, and also called *edg-2*/*mrec*/*Gpcr26*) increased responsiveness to LPA in cell rounding and adenylate cyclase inhibition assays when overexpressed in cerebral cortical cell lines (Hecht et al., 1996). Heterologous expression of LP_{A1} in hepatoma (RH7777) and neuroblastoma (B103) cell lines which have no endogenous responses to LPA further demon-

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Abbreviations: *Cnr1* and *Cnr2*, approved symbols for the murine CB1 and CB2 cannabinoid receptor genes; *Edg-1*, approved symbol for endothelial differentiation gene-1; FCS, fetal calf serum; GCG, Genetics and Computer Group; GPCR, G-protein coupled receptor; GTPγS, guanosine-5′-O-(3-thio)-triphosphate; kb, kilobase(s); LPA, lysophosphatidic acid; MAP kinase, mitogen activated protein kinase; pBS, pBluescript; S1P, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine; SRE, serum-response element; TMD, transmembrane domain; www, world-wide web.

[1] When referring to a protein, uppercase is used (e.g. LP_{A1}, EDG-1). When referring to a gene, we utilize the same symbol as in the original publication (lp_{A1}, edg-3, H218), with the exception of *Gpcr26* and *Edg-1*, which are symbols approved by the Mouse Genome Database.

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strated that this receptor is sufficient in conferring specific ^{3}H-LPA binding and multiple LPA-dependent responses (Fukushima et al., 1998), consistent with its initial identification as a receptor for LPA. Independent support for this identity was obtained by expression of LP_{A1} in either yeast (Erickson et al., 1998) or lymphoid cells (An et al., 1997b), in each case conferring or potentiating responses to LPA.

We hypothesized that orphan receptor genes with substantial amino acid sequence similarity to LP_{A1} could encode additional lysophospholipid receptors. Amino acid identities amongst members of other GPCR subfamilies which recognize the same ligand are generally above 35%. Three orphan GPCRs isolated from human or rat (*Edg-1*, H218/AGR16, and edg-3) shared 32–36% identity with LP_{A1}, and were thus candidate lysophospholipid receptors. *Edg-1* was initially isolated in a subtractive screen for genes induced upon human endothelial cell differentiation (Hla and Maciag, 1990). H218 and AGR16 (names given independently to the same rat gene) were isolated by low-stringency screening with a D2 dopamine receptor probe or by degenerate PCR from rat aortic smooth muscle, respectively (Okazaki et al., 1993; MacLennan et al., 1994), while edg-3 was isolated from human genomic DNA using degenerate cannabinoid receptor primers (Yamaguchi et al., 1996).

Murine homologs of *Edg-1*, H218/AGR16, and edg-3 could be useful in the study of lysophospholipid receptors. First, receptor clones from the same organism can be studied comparatively in terms of sequences, expression patterns, genomic structures, and chromosomal localizations. Second, heterologous expression of receptors from the same species could allow comparative functional studies of ligand specificity and efficacy. Third, murine clones would provide necessary reagents for producing receptor-null mice. Recently, several groups have reported that non-murine receptors encoded by *Edg-1*, H218/AGR16, and edg-3 are S1P receptors (An et al., 1997a; Lee et al., 1998b; Zondag et al., 1998). For *Edg-1*, S1P induces Ca^{++}-dependent changes in cell morphology, induction of P-cadherin mRNA, increases MAP kinase activity, and internalization of the *Edg-1* receptor (Lee et al., 1998b; Zondag et al., 1998). For H218 and edg-3, S1P induces serum-response element (SRE) activation and changes in Ca^{++} flux (An et al., 1997a). None of the previous studies showed that receptor expression conferred S1P responsiveness in the form of specific and direct G-protein activation.

Here we show that the three identified murine receptors can be grouped in a subfamily distinct from the lp_A LPA receptor genes. The coding regions of each are contained in a single exon. Each receptor confers S1P-inducible G-protein activation when heterologously expressed in a cell line that is unresponsive to S1P or LPA. Overall, expression patterns are distinct, both in the embryo and adult, although overlapping expression can be detected in some individual tissues. These three genes thus appear to encode a subfamily of differentially expressed S1P receptors.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Sigma with the following exceptions (or otherwise noted): 1-oleoyl lysophosphatidic acid (Avanti Polar Lipids), sphingosine-1-phosphate (Biomol), lipofectamine transfection reagent and cell culture media (Gibco BRL), random hexamers, Klenow DNA polymerase, and digoxigenin-UTP labeling mix (Boehringer Mannheim), restriction enzymes (New England Biolabs), pFlag·CMV-1 (Kodak), pBluescript (Stratagene), ^{35}S-GTPγS, ^{32}P-dCTP, and ^{35}S-dATP (NEN Life Science Products), SeaKem LE and SeaPlaque GTG agarose (FMC Bioproducts). Oligonucleotides were synthesized with an Expedite Nucleic Acid Systems machine (Millipore). Deionized water was purified using a Millipore filtration system. RH7777 hepatoma cells were obtained from Hyam Leffert (University of California, San Diego, CA); B103 neuroblastoma cells from David Schubert (Salk Institute, La Jolla, CA); and C6 glioma cells from ATCC, Inc.

2.2. PCR reactions

PCR was used to specifically amplify fragments from the murine genes corresponding to *Edg-1*, H218/AGR16, and edg-3. Oligonucleotides for *Edg-1* and H218/AGR16 were designed to either murine expressed sequence tags deposited in GenBank [accession numbers AA145076 (edg1a), and L20334 (edg4a, edg4b)] or to published rat sequence (edg1b; Lado et al., 1994). [The murine *Edg-1* DNA sequence (Liu and Hla, 1997) was not available at the time experiments were initiated]. For edg-3, only the human sequence was known. In order to minimize mismatches between human edg-3 primers and the corresponding murine gene, oligonucleotides were designed to coding regions that encoded highly conserved 6–8 amino acid stretches. Primer pairs that worked well and corresponding product sizes were: *Edg-1* (681 bp): edg1a (5′-GGG ACA CAA TTA GCA GCT AT-3′, sense) and edg1b (5′-GTA GAG GAT GGC GAT GGA AAG-3′, anisense); H218/AGR16 (416 bp): edg4a (5′-TTA-ACT CCC GTG CAG TGG TTT GC-3′, sense) and edg4b (5′-ACG ATG GTG ACC GTC TTG AGC A-3′, antisense); edg-3 (681 bp): edg3a (5′-CGC ATG-TAC TTT TTC ATT GGC AA-3′, sense) and edg3c (5′-GGG TTC ATG GCG GAG TTG AG-3′, antisense). Oligonucleotides were purified by polyacrylamide gel-electrophoresis and then with Sep-Pak C18 reverse phase cartridges (Waters) (Ausubel et al., 1994). PCR reaction mixes consisted of 1× PCR buffer B [15 mM $(NH_4)_2SO_4$, 75 mM Tris pH 8.5, 2.0 mM $MgCl_2$],

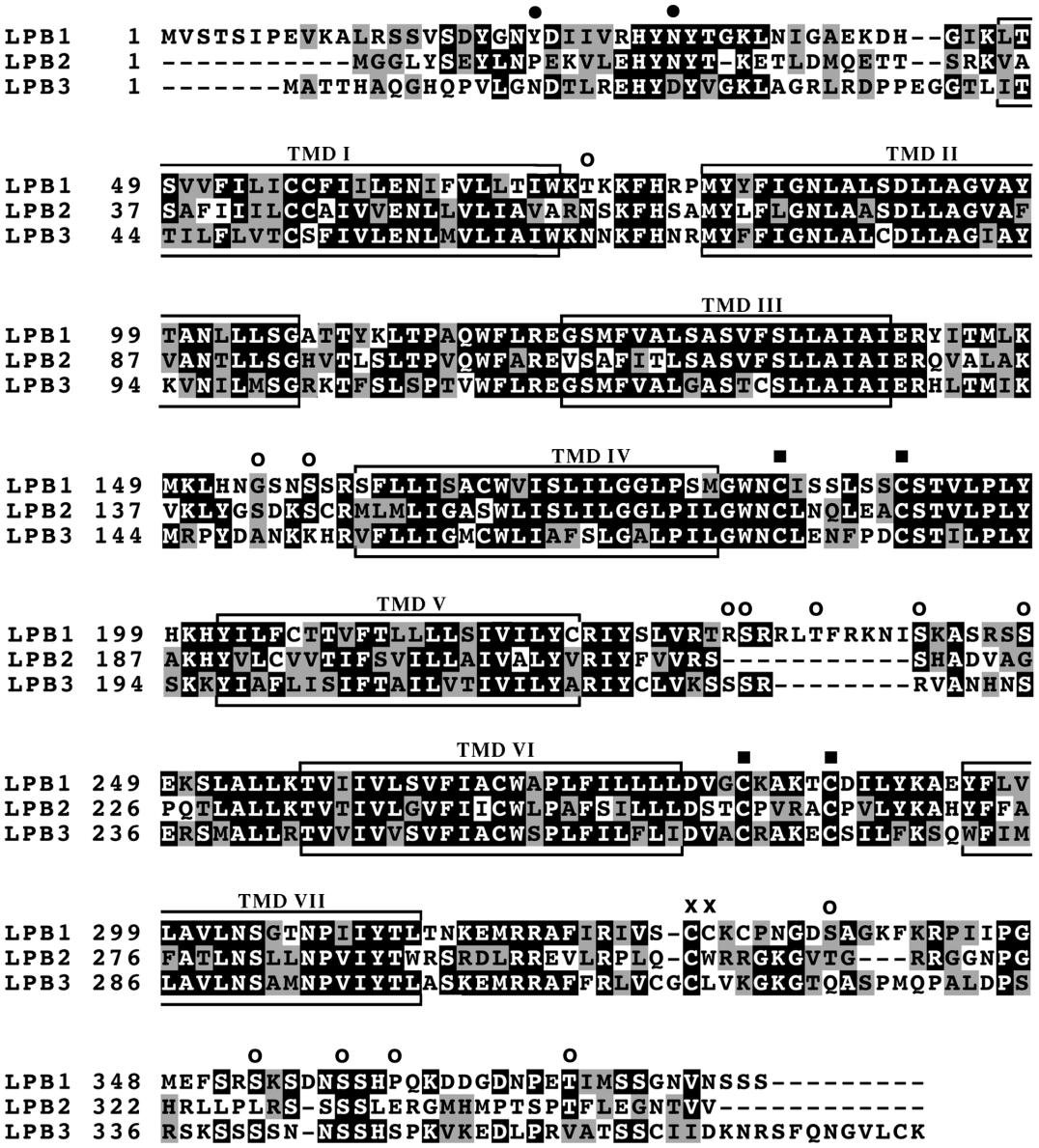

Fig. 1. Alignment of predicted amino acid sequences for murine LP_B receptors. Residues identical in two or three of the sequences are shown in white on a black background, while conservative changes are shaded in gray. Approximate locations of the seven putative transmembrane domains (TMDs) are bracketed (Hecht et al., 1996). Sites of putative post-translational modifications (see methods) in one or several of the receptors are indicated: N-linked glycosylation (●), serine or threonine phosphorylation (○), cysteine palmitoylation (×), and cysteines involved in disulfide bonds (■). Kinases corresponding to phosphorylation consensus sequence sites are protein kinase A (R/K, R/K, −, T), protein kinase C (S/T, −, R/K), and casein kinase II (S/T, −, −, D/E). Note that in the last five indicated potential phosphorylation sites, consensus sequences are only present on one of the aligned proteins. A consensus leucine zipper motif exists from amino acids 47–68 (TMD I) in LP_{B1}. The lp_B ORF DNA sequences have been deposited with GenBank. lp_{B1}: Genbank Accession Number AF108019, lp_{B2}: Genbank Accession Number AF108020, lp_{B3}: Genbank Accession Number AF108021.

0.5 μM each primer, and 0.25 mM each dNTP. In general, after addition of template (5% of the reaction volume), the mix was overlaid with mineral oil, heated to 90°C, and Taq (0.02 U/μl) added before cycling 35× (94°C, 30 s; 54°C, 30 s; 72°C, 2 min), with a final extension step of 10 min at 72°C. When screening phage or bacterial samples, the order of addition of Taq and template was sometimes reversed.

2.3. Isolation of genomic clones

A mouse 129SvJ genomic library (Stratagene) was screened using a PCR protocol detailed in Contos and Chun (1998). PCR products were visualized by electrophoresis on 1.4% agarose gels containing ethidium bromide. Because the reactions generally only gave a single positive product of the expected size, visualization by

ethidium bromide was all that was necessary to identify wells containing positive clones. For final screens, approximately 500 phage from positive wells were plated out on 10 cm dishes, and conventional nylon filter lifts were made (Ausubel et al., 1994). These were screened with ^{32}P-labeled DNA probes (synthesized using random hexamers) prepared from qiaex gel-purified (Qiagen) PCR products of each respective lp_B gene. Pure, isolated, positive plaques were cored out, grown by liquid lysate, and prepped using the Wizard λ DNA Miniprep kit (Promega).

2.4. Subcloning, sequencing, and sequence analysis

DNA from each λ genomic clone was analyzed by restriction mapping and southern blotting (Ausubel et al., 1994). PCR products (edg1ab, edg4ab, and edg3ac) were T/A cloned into pBluescript (pBS) that was digested with *Eco*RV and treated with Taq + dTTP (Marchuk et al., 1991). Genomic λ DNA fragments containing the open reading frames were subcloned into pBS and sequenced by the dideoxy method using primers flanking the multiple cloning site and gene-specific primers synthesized for the purpose. Protein alignments were made using the CLUSTAL W application program on the www (http://www.ibc.wustl.edu/ibc/msa.html). Disulfide bonds between two conserved cysteines on extracellular loops 2 and 3 are a common feature of many GPCRs (Watson, 1994). In addition, cysteines on the intracellular C-terminal tail have been shown to be palmitoylated in several GPCRs (Watson, 1994; Morello and Bouvier, 1996). Protein sequences were analyzed using the PROSITE database on the www (http://pbil.ibcp.fr/NPSA/npsa_prosite.html). In Fig. 1, we do not indicate several consensus sequences for the following reasons: N-myristoylation (it is not known if LP_B receptors are proteolytically processed at their N-termini), phosphorylation (if located on putative TMD or extracellular domains), glycosylation (if located on putative TMD or intracellular domains), and amidation (all sites were located intracellularly).

2.5. Expression construct cloning

Expression constructs were made in the pFlag · CMV-1 vector. To create pFlag · lp_{B1}, a subcloned genomic fragment was digested with *Bst*XI (which cleaves 20 bp upstream of the start codon), blunted using T4 DNA polymerase, heat-inactivated, and cleaved with *Bam*HI (recognizing a site in the vector). This 1.4 kb fragment was cloned into pFlag · CMV-1 that was cleaved with *Not*I, filled-in with Klenow, heat-inactivated, and cleaved with *Bam*HI. For lp_{B2}, an *Nco*I site exists at the ATG start codon. After *Nco*I digestion, the plasmid was filled-in with Klenow, heat-inactivated, and a *Sal*I site (in vector sequence) cleaved 1.7 kb downstream. This fragment was cloned into pFlag · CMV-1 digested with *Eco*RV and *Sal*I. Because lp_{B3} also has an *Nco*I site at the ATG start codon, it was treated similarly to lp_{B2}, except a *Bgl*II site 1.2 kb downstream was used as the 3′ cutter. This fragment was cloned into pFlag · CMV-1 that was digested with *Hind*III, filled-in, heat-inactivated, and further digested with *Bam*HI. Cloning junctures at the 5′ end of the open reading frame in each expression construct were confirmed by sequencing.

2.6. [^{35}S]-GTPγS binding assay

RH7777 hepatoma cells approximately 50% confluent were transfected in DMEM alone with lipofectamine (Gibco BRL) complexes of control pFlag · CMV-1 or experimental vectors (pFlag · lp_{B1}, pFlag · lp_{B2} or pFlag · lp_{B3}) for 15 h, washed, then fresh serum-free DMEM (with 1 × penicillin/streptomycin, i.e. 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate) added. After incubation for 30 h, membrane fractions were prepared by homogenization in ice-cold TED buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT) containing 1 × protease inhibitor cocktail, centrifugation at 1000***g*** for 5 min at 4°C to remove nuclei, and further centrifugation at 15,000***g*** for 20 min at 4°C. Membranes were kept at 4°C after resuspension in GTP-binding buffer (50 mM Hepes-NaOH, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT), proteins quantified using the Bradford assay (Ausubel et al., 1994), then 25 μg protein equivalents of membrane incubated 30 min at 30°C with either LPA or S1P (diluted in 0.1% fatty acid-free BSA) in GTP-binding buffer containing 10 μM GDP and 0.1 nM [^{35}S]-GTPγS (1200 Ci/mmol). Unbound [^{35}S]-GTPγS was separated by passage through GF/C filters, while bound [^{35}S]-GTPγS was eluted in scintillation fluid and quantitated in a scintillation counter. Specific binding of [^{35}S]-GTPγS was determined by subtracting the quantity obtained by concurrent incubation with 100 μM GTPγS. To be certain our constructs led to protein expression, transfected cells were fixed, stained with anti-Flag M2 antibody, and visualized by indirect immunofluorescence using fluorescein isothiocyanate (as in Fukushima et al., 1998).

2.7. Northern hybridization analysis

Adult C57BL/6J mice were sacrificed by cervical dislocation and organs immediately dissected, frozen in liquid nitrogen, and stored at −80°C. RH7777, B103, and C6 cell lines were maintained in DMEM supplemented with 10% fetal-calf serum (FCS) and 1 × penicillin/streptomycin. TR and TSM cell lines were maintained in OptiMEM supplemented with 2.5% FCS and 1 × penicillin/streptomycin. Total RNA was prepared and northern blotting performed using standard protocols (Ausubel et al., 1994). Blots were stained with

methylene blue and photographed as a quantitative control. For probe preparation, T/A cloned inserts of PCR products (edg1ab, edg4ab, or edg3ac) were excised from their respective plasmids with *Eco*RI and *Xho*I, gel-purified using the qiaex gel-extraction kit (Qiagen), and labeled with ^{32}P by random hexamer priming. Blots were incubated at 55°C overnight with 5×10^6 cpm/ml in hybridization solution (25% formamide, 0.5 M Na_2HPO_4, 1% BSA, 1 mM EDTA, 5% SDS), followed by successive 20 min RT washes in 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, 0.5× SSC/0.1% SDS, 0.2× SSC/0.1% SDS, and a final wash with 0.2× SSC/0.1% SDS at 65°C.

2.8. In situ hybrydization

Plasmids containing T/A cloned PCR products (pBS-edg1ab, pBS-edg4ab, and pBS-edg3ac) were linearized with either *Xho*I or *Bam*HI and sense or antisense digoxigenin-labeled riboprobes synthesized with T3 or T7 RNA polymerase according to manufacturer's protocols (Boehringer Mannheim). Cryostat sections (20 μM) were processed and hybridized as previously described (Weiner and Chun, 1997). As a control, sections adjacent to those used for antisense riboprobes were hybridized with sense riboprobes, which never produced any significant labeling.

3. Results

3.1. Characterization of murine genomic clones for lp_{B1}, lp_{B2}, and lp_{B3}

To isolate murine genomic clones for *Edg-1*, H218/AGR16, and edg-3, oligonucleotides were designed based upon expressed sequence tags (*Edg-1* and H218/AGR16) or conserved regions of the corresponding human (edg-3) or rat gene (*Edg-1*). PCR reactions yielding a single product of the expected size from murine genomic DNA were used to screen a mouse 129SvJ genomic library. PCR products and genomic fragments were subcloned and sequenced. From this analysis we determined that each of the open reading frames was encoded within a single exon, unlike the two characterized lp_A genes, where an intron is inserted in the middle of the coding region for transmembrane domain VI (TMD VI; Contos and Chun, 1998). We termed the murine genes lp_{B1} (*Edg-1*), lp_{B2} (H218/AGR16), and lp_{B3} (edg-3) for lysophospholipid ('*lp*') receptors of a second ('B') subclass (Fig. 1). Each of the receptors has seven putative transmembrane domains, as well as putative glycosylation, phosphorylation, and palmitoylation sites. In all LP_B proteins there are two cysteines in each of the extracellular loops 2 and 3 that can potentially form disulfide bonds (Fig. 1), as found in other GPCRs (Watson, 1994). Without post-translational modifications, the predicted molecular weights of LP_{B1}, LP_{B2}, and LP_{B3} polypeptides are 42571 Da, 38871 Da, and 42270 Da, respectively (Fig. 1). Pairwise alignments were made between the predicted amino acid sequences from our murine clones and non-murine homologs. In general, corresponding rat, human, and murine amino acid sequences of the same receptor were >87% identical to one another (Fig. 2). Different receptor pairs (i.e. LP_{B1}/LP_{B2}, LP_{B1}/LP_{B3}, or LP_{B2}/LP_{B3}) within the same organism or between organisms shared 44–52% identity overall, which is greater than the identity between any LP_B vs. LP_A protein (32–36% identity). Thus, the lp_B genes appear to occupy a distinct receptor gene subfamily compared with that of the lp_A and *Cnr* (cannabinoid receptor) genes.

3.2. Each LP_B receptor confers S1P-dependent G-protein activation in RH7777 cells

The possibility that lysophospholipid ligands activate LP_B receptors was investigated by heterologous expression of each receptor in a cell line followed by measurement of G-protein activation in response to various ligands. We chose RH7777 hepatoma cells because they did not respond to the lysophospholipid ligands LPA or S1P in various assays (Fukushima et al., 1998 and data not shown), and also did not express any of the lp_A or lp_B genes (see next section). The plasmids pFlag·lp_{B1}, pFlag·lp_{B2} and pFlag·lp_{B3} were constructed by in-frame ligation of open reading frames of each respective lp_B gene into the pFlag·CMV vector, producing a receptor containing a 'FLAG' N-terminal epitope. Tagged receptors were functionally indistinguishable from native receptors based on multiple assays (data not shown). Approximately 20% of the cells transiently transfected expressed the Flag-tagged receptor (Fig. 3A). G-protein activation was measured by incubation of membrane fractions with potential lysophospholipid ligands and [^{35}S]-GTPγS, a poorly hydrolyzable analog of GTP. We have previously shown that LPA will activate G-proteins in this assay through LP_{A1} (Fukushima et al., 1998). Control empty vector-transfected RH7777 cells did not increase G-protein activation in response to LPA or S1P at concentrations up to 10 μM (Fig. 3B). However, when any of the LP_B receptors were expressed individually, significant, dose-dependent increases in G-protein activation were observed in response to S1P, but not to LPA (Fig. 3B). Dose-response curves demonstrated increasing [^{35}S]-GTPγS binding with increasing concentration of S1P, up to a saturation point around 1 μM (Fig. 3C).

3.3. lp_B genes are differentially expressed in adult mouse tissues and cell lines

To determine the comparative mRNA expression patterns of the lp_B genes in various adult mouse tissues,

A

% AMINO ACID IDENTITY

	LP_{B1m}	EDG-1r	EDG-1h	LP_{B2m}	H218r	LP_{B3m}	EDG-3h	LP_{A1m}
LP_{B1m}		92.9	97.4	47.4	47.7	50.0	51.9	35.7
EDG-1r	97.6		92.7	48.3	48.6	50.7	51.9	35.6
EDG-1h	94.5	94.2		46.9	47.1	49.6	51.1	35.6
LP_{B2m}	58.0	58.6	59.1		96.6	44.3	46.1	32.2
H218r	58.0	58.6	59.1	97.2		44.6	46.3	32.4
LP_{B3m}	60.5	59.7	61.6	53.4	53.7		87.5	34.5
EDG-3h	61.7	60.9	62.0	55.0	54.9	90.2		35.0
LP_{A1m}	44.8	44.8	44.7	40.7	41.3	43.8	43.6	

% AMINO ACID SIMILARITY

Fig. 2. Sequence similarity of LP_B receptors. Table of % amino acid identity (in the upper right half) or % amino acid similarity (in the lower left half) of LP_B receptors from mouse or the corresponding proteins from rat (EDG-1r, H218r) or human (EDG-1h, EDG-3h). For comparison, the LP_{A1} receptor is also shown. Homologs of the same gene from different organisms are shaded. Values were determined by alignment of entire protein sequences using GCG Gap (version 9.1).

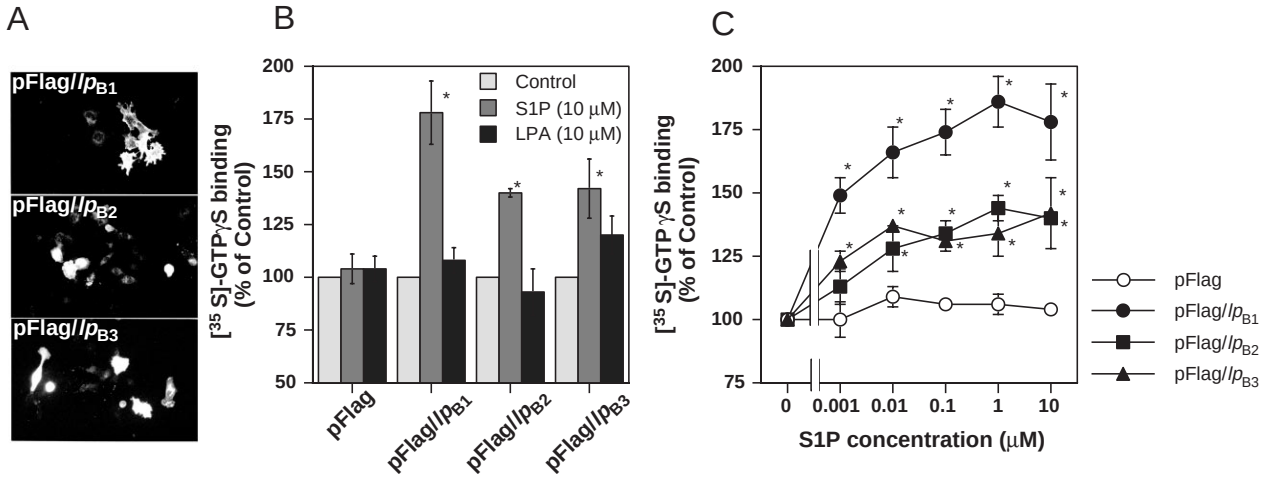

Fig. 3. Ligand-specificity and S1P dose-response of G-protein activation in LP_B-transfected RH7777 cells. (A) Immunofluorescence labeling using the anti-Flag M2 antibody on RH7777 cells transiently transfected with constructs pFlag·lp_{B1}, pFlag·lp_{B2}, or pFlag·lp_{B3}. (B) Amount of specific [^{35}S]-GTPγS bound to the membranes treated with vehicle solution, 10 μM LPA, or 10 μM S1P. Basal [^{35}S]-GTPγS binding (fmol/mg protein) was 22.9±8.3 (pFlag), 26.5±3.8 (pFlag·lp_{B1}), 17.6±3.4 (pFlag·lp_{B2}), and 30.1±3.9 (pFlag·lp_{B3}). Specific binding activity in treatment groups was determined relative to vehicle solution only. Data are mean±SEM ($n \geq 3$). (C) Dose-response curve for S1P in LP_B-transfected RH7777 cells. Basal [^{35}S]-GTPγS binding (fmol/mg protein) was 20.0±3.1 (pFlag), 20.9±4.2 (pFlag·lp_{B1}), 22.5±2.0 (pFlag·lp_{B2}), and 30.7±5.2 (pFlag·lp_{B3}). Data are mean±SEM ($n \geq 3$). Treatments significantly different ($p < 0.05$; Student's T-test) from vehicle-only control are indicated with an asterisk (*).

northern blots were probed at high stringency with each lp_B coding region (Fig. 4). The ~3.0 kb lp_{B1} mRNA was expressed highly in brain, heart, lung, liver, and spleen; moderately in kidney, thymus, and muscle; and negligibly in testis, stomach, and intestine. In contrast, the ~2.8 kb lp_{B2} mRNA was most abundant in heart and lung; low, but clearly observed in kidney, liver, and thymus; and much lower but detectable in the other tissues examined. There was also a smaller ~1.0 kb transcript in thymus, expressed at approximately the same level as the larger ~2.8 kb transcript. The ~3.8 kb lp_{B3} transcript was most abundant in heart, lung, kidney

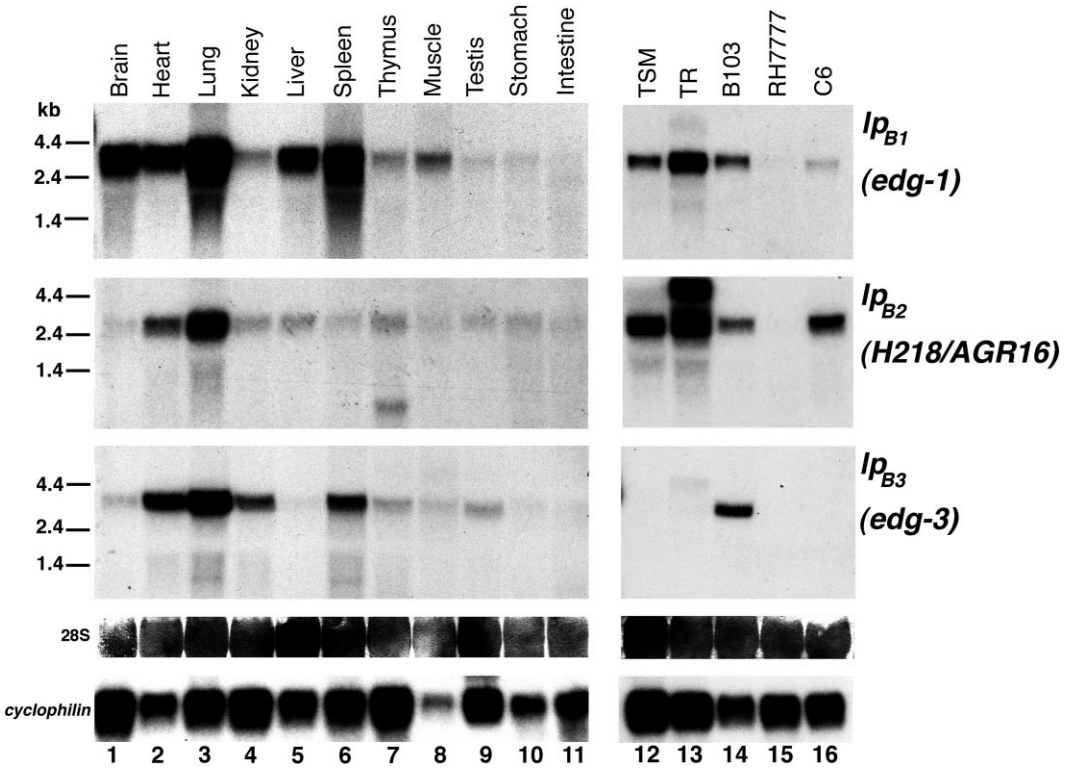

Fig. 4. Expression analysis of lp_B genes by northern blot. Total RNA (20 μg per lane) from indicated sources was blotted and probed at high stringency with DNA fragments specific for coding regions of lp_{B1}, lp_{B2}, or lp_{B3}. To control for variability in RNA quantity between lanes, the blot was stained with methylene blue, which stains all RNA (only the 28S rRNA band is shown), and/or also hybridized with a murine cyclophilin cDNA probe, which is ubiquitously expressed, albeit at varying levels. Positions of molecular weight markers are indicated on the left. All tissue blots were exposed for 4 days, while those for cell lines were exposed only 24 h.

and spleen; low but detectable in brain, thymus, muscle and testis; and nearly undetectable in liver, stomach, and intestine. Overall, heart and lung express all of these receptor genes at relatively high levels, whereas testis, stomach, and intestine express relatively little of any of these receptor genes.

Northern blots were also analyzed to determine the comparative expression of the lp_B genes in several cell lines. TR and TSM are embryonic cerebral cortex-derived cell lines that were initially used in isolation of lp_{A1} (Chun and Jaenisch, 1996; Hecht et al., 1996). Both the neuroblastoma B103 and hepatoma RH7777 cell lines were previously shown to be unresponsive to LPA treatment (Fukushima et al., 1998). C6 glioma cells are widely used in studies of glial cell functions. In general, each cell line expressed one or more of the lp_B transcripts with the exception of RH7777, which expressed none at detectable levels, consistent with its lack of response to S1P. There was a larger transcript size of ~5 kb for lp_{B2} in the TR cell line, in addition to the more common ~2.8 kb transcript.

3.4. In situ hybridization

To determine the comparative spatial expression patterns of each lp_B gene in embryonic day (E) 14–18 embryos, in situ hybridization was used. Though each of the lp_B genes was expressed highly in adult lung (see above), only lp_{B3} was expressed in embryonic lung from E14–E18 (Fig. 5 and data not shown). The lp_{B3} transcript was also abundantly detected in embryonic nasal cartilage, sphenoid bone, vena cava, Meckel's cartilage/incisor teeth, genital tubercle, and bladder (Fig. 5 and data not shown). The lp_{B1} transcript was expressed modestly in apparent endothelial cells surrounding some blood vessels (e.g. aortic trunk, Fig. 5A). While low expression in regions of ossification was observed as previously reported (Liu and Hla, 1997), expression of lp_{B3} was often much more prominent (cf. Fig. 5H) in the same structure. The lp_{B2} transcript was not significantly detected in any of the sections, except embryonic brain, where each of the lp_B transcripts was expressed to some degree (J.A. Weiner and J. Chun, unpublished observations and in preparation).

4. Discussion

We have isolated complete murine genomic coding sequences of three genes with substantial amino acid identity to the first identified lysophospholipid receptor, LP_{A1}. Based on their sequence similarity and S1P ligand

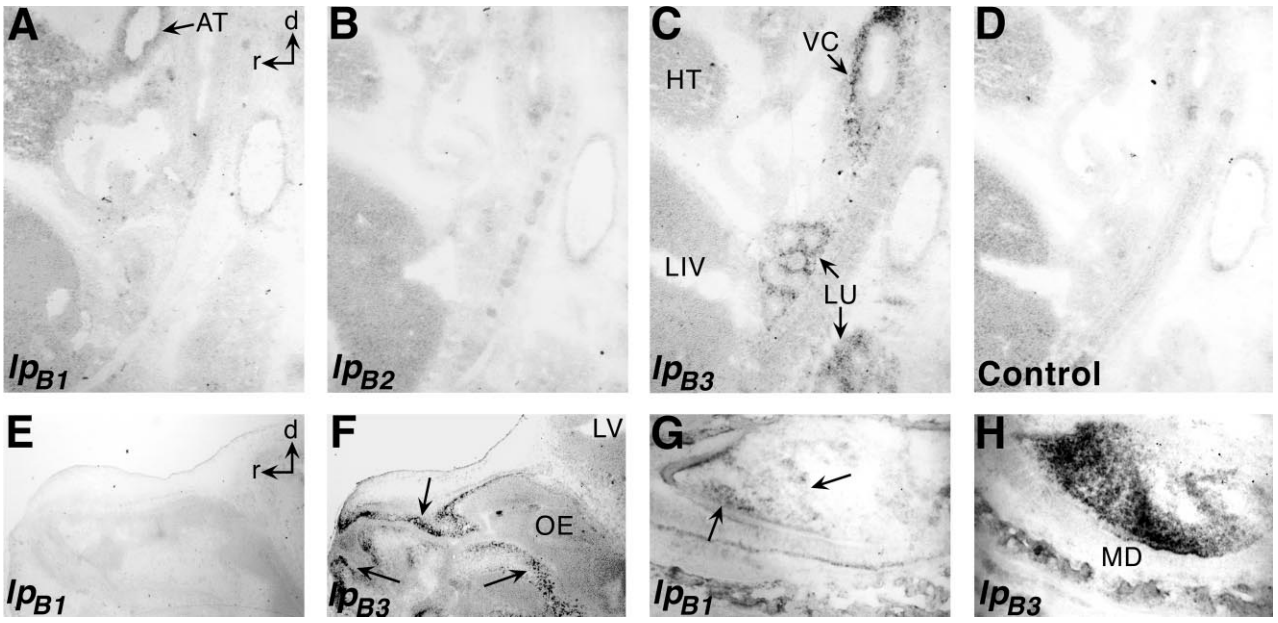

Fig. 5. Expression analysis of lp_B genes by in situ hybridization. The indicated digoxigenin-labeled sense (D) or antisense riboprobes (A–C, E–H) were hybridized to mouse embryonic day 14 (A–F) or embryonic day 18 (G–H) sagittal sections. Sections A–D are mid-body, E–F are rostral head, and G–H are ventro-rostral head. Arrows point to areas of expression for each respective lp_B gene; in F, these are nasal cartilage primordia, while in G and H these are Meckel's cartilage and/or primordium of incisor teeth. The lp_{B3} transcript is also expressed through E18 primordial lung and in E14–E18 genital ridge (not shown). The lp_{B2} transcript was not detected significantly in any of these sections (shown only in B). Orientation is indicated (d = dorsal, r = rostral). AT, aortic trunk; HT, heart; LIV, liver; VC, vena cava; LU, lung; OE, olfactory epithelium; LV, lateral ventricle; MD, mandibular bone.

responsivity, these receptors are logically placed in a separate gene subfamily. Because several published names for lysophospholipid receptors are non-systematic and confusing (e.g. *Edg-2* and *Edg-3* have been used for non-GPCR genes isolated in the same screen as *Edg-1*; Hla et al., 1995; Hla et al., 1997, and the non-murine homologs include the names *Edg-1*, H218/AGR16, and edg-3), we refer to our murine clones as lysophospholipid ('*lp*') receptors of a second 'B' subclass. This name also accounts for the observation that EDG-1/LP_{B1} appears to be a low-affinity receptor for another lysophospholipid, LPA (Lee et al., 1998a) as well as a high-affinity receptor for S1P. The three lp_B genes were comparatively examined in terms of sequence, ligand responsiveness, and expression patterns.

The overall amino acid identity among all LP_B members is 44–50%, though if one aligns only the TMDs, it is 55–64%. This result is not unexpected, since the amino acids conserved amongst most GPCRs are located within TMDs, and ligand-recognition sites appear to be located here (Schwartz, 1994). Outside of the TMDs there is 35–39% identity. In other GPCRs, domains such as the third intracellular loop (i_3; located between TMDs V and VI) and C-terminal tail are known to specify G-protein coupling as well as to contain phosphorylation sites critical for desensitization (Ferguson et al., 1996; Wess, 1997). Relative to the LP_{B1} receptor, LP_{B2} and LP_{B3} have 11 and eight amino acids deleted in i_3, respectively. Divergence in intracellular domains may allow LP_B receptors to couple to distinct Gα subunits, as well as provide a basis for feedback regulation by distinct kinases. To date, G-protein coupling studies have only been published for *Edg-1*, whose i_3 region was shown to interact with Gαi1 and Gαi3 in a ligand-*independent* manner (Lee et al., 1996). These data are consistent with other studies demonstrating that S1P acts through Gi (van Koppen et al., 1996). However, S1P is known to also act through non-Gi proteins (Yamamura et al., 1997), including the small GTPase Rho (Postma et al., 1996), which may be activated through Gα13-, Gαq-, or Gα12-dependent pathways (Sah et al., 1996; Fromm et al., 1997; Gohla et al., 1998). Thus, LP_B members likely couple to several types of G-proteins, just as has been observed for the LPA receptor (Fukushima et al., 1998). In addition to G-protein coupling, distinct LP_B receptors may also couple to other proteins. In support of this hypothesis is the presence of a putative Src-homology 2 (SH2) domain (identified by sequence similarity) in H218/AGR16 and LP_{B2} (MacLennan et al., 1994), which is not present in either LP_{B1} or LP_{B3} (data not shown), and could theoretically mediate binding to phosphotyrosines on partner proteins.

By isolating the lp_B members from genomic DNA, we determined that each lacks introns within its coding region, consistent with prior data on murine *Edg-1* (Liu and Hla, 1997) and human edg-3 genes (Yamaguchi et al., 1996), and providing new support for the place-

ment of the H218/AGR16 gene in this subfamily. The next closest GPCR subfamily (the lp_A gene subfamily) contains a conserved intron within the middle of the coding region for TMD VI, which evolutionarily appeared after divergence from the lp_B subfamily (Contos and Chun, 1998). A more detailed analysis of the genomic structure, cDNA sequences, transcription start sites, and chromosomal localizations of lp_B genes will be presented elsewhere (G. Zhang, J.J.A. Contos, and J. Chun, in preparation).

Heterologous expression data shown here support the hypothesis that each of the three LP_B receptors specifically recognizes the lysophospholipid ligand S1P, activating G-proteins in response to ligand stimulation. These data complement competitive binding experiments for EDG-1/LP_{B1} (Lee et al., 1998b) and provide direct evidence for S1P-dependent G-protein activation for all three receptors, as compared with assays dependent on downstream signaling pathways, such as SRE or MAP kinase activation (An et al., 1997a; Lee et al., 1998b; Zondag et al., 1998). The use of a single, downstream reporter assay likely explains the lack of S1P-dependent responsiveness observed for EDG-1/LP_{B1} (An et al., 1997a) as compared with examination of more proximal portions of S1P signaling shown here and previously (Lee et al., 1998b; Zondag et al., 1998).

It is possible that other S1P-like ligands could also act on the LP_B receptors. There are a range of chemical derivatives present in cells that have core sphingosine structure, including sphingosylphosphorylcholine (SPC), ceramides (N-acyl sphingosines), ceramides with various groups attached to the 1-hydroxyl (cerebrosides, globosides, gangliosides, sulfatides), and sphingomyelin (N-acyl-sphingosine-1-phosphorylcholine). We did observe (for each LP_B receptor) weak responsivity to both sphingosine and SPC (data not shown), however, the extent to which sphingosine is converted to S1P by the enzyme sphingosine kinase (present intracellularly and probably on our membrane preparations) was not determined, and could explain the observed activity (Meyer zu Heringdorf et al., 1997; Yatomi et al., 1997). The identity of endogenous, biologically relevant ligands for the LP_B family thus remains an open question.

The LP_B receptors were not significantly activated by LPA, which suggests that the LP_B subfamily can discriminate between the two ligands at the analyzed ligand concentrations and using G-protein activation as an assay. Though the S1P-specificity is consistent with several reports (Postma et al., 1996; An et al., 1997a; Lee et al., 1998b; Zondag et al., 1998), these data conflict with another report that S1P and LPA can act through the same receptor, based on competitive binding and cross-desensitization experiments in a human platelet aggregation assay (Yatomi et al., 1997). The cross-desensitization could theoretically be due to desensitization of a common intracellular pathway by both S1P and LPA receptors (e.g. the same kinase phosphorylating both LP_A and LP_B after the aggregation pathway is underway), although this would not explain the competitive binding results. Perhaps under certain circumstances and specific cell types, both S1P and LPA could activate the same LP_B receptor(s), as recently reported for Edg-1 (Lee et al., 1998a).

Our expression data demonstrate that some tissues can express none, one, two, or all three of the lp_B transcripts. Our data contrast in several instances to prior reports. AGR16 was reported as most abundant in rat lung and heart (Okazaki et al., 1993), as we confirm for mouse, but we have not observed the moderate expression levels reported in stomach and intestine. Human edg-3 had the relative expression levels heart > kidney > liver > muscle > lung, while we observed lung > heart > kidney > muscle, and little if any detection in liver. With lp_{B1}/*Edg-1*, we obtained results consistent with those previously published for mouse (Liu and Hla, 1997), and partly consistent with what was observed in rat (Lado et al., 1994). In mouse, high levels were found in lung with moderate levels in heart and liver, while in rat, very low levels were observed in lung, heart, and liver. The discrepancies in expression between mouse, rat, and human could simply be species-specific differences or may reflect particular ages or states that the animals are in at the time of RNA isolation. Novel findings reported here include the overlapping expression of lp_B genes in several different murine tissues, most notably lung and heart, and embryonic expression of lp_{B3}.

There are several potential roles for multiple receptor subtypes in the same tissue. Distinct cell types within a single tissue may express different lp_B genes to allow different responses to a single ligand for those cell types. If the lp_B genes couple to different downstream pathways, as suggested by the presence of an SH_2 domain in LP_{B2}, then S1P could have very different effects on a given cell type. Alternatively, more than one lp_B gene could be expressed in the same cell, resulting either in redundancy of function, or synergistic responses to S1P stimulation (based on divergent G-protein coupling or related signaling). Our data showing expression of lp_B receptor genes in most tissues are consistent with the fact that S1P influences many cell types (Meyer zu Heringdorf et al., 1997; Spiegel et al., 1998). In addition, the finding that several embryonic tissues express lp_B transcripts suggests that S1P has a role in development, which has not been addressed previously.

The comparative analysis of the murine lp_B receptor subfamily indicates that each gene encodes a GPCR that responds to S1P, but not LPA, through the activation of G-proteins. Each gene shows strong sequence and genomic structural similarities, yet distinguishable spatial and temporal expression patterns that suggest distinct biological roles through the operation of one or

more receptors at a given age or tissue. These data form a foundation from which the biological effects of genetic perturbation of the lp_{B} family in the mouse model system can be anticipated and interpreted in future studies.

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