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Characterization of a Zebrafish (*Danio rerio*) Sphingosine 1-Phosphate Receptor Expressed in the Embryonic Brain

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Sphingosine 1-phosphate elicits a variety of responses in mammals via at least five G protein-coupled Edg receptors. We cloned zebrafish *edg1* and expressed it in Rh7777 cells. In these cultures, S1P inhibited forskolin-driven rises in cAMP and this response was eliminated by pretreatment of the cultures with pertussis toxin. In Rh7777 membranes, S1P stimulated GTPγ[^{35}S] binding 2–3 fold. Zebrafish *edg1* is expressed in embryonic brain, particularly ventral diencephalon, optic stalks, and anterior hindbrain. Our findings suggest that nonmammalian vertebrates use S1P to signal during embryogenesis and that the properties of Edg1 receptor have been conserved for 400 million years.

Key Words: **zebrafish; Edg1; sphingosine 1-phosphate; G protein-coupled receptor; signal transduction; evolution.**

Sphingosine 1-phosphate (S1P), a metabolite of sphingosine, is one of the biologically active lysophospholipids that evokes a variety of cellular responses, including cell proliferation, anti-apoptosis, and neurite retraction (1–3). The actions of S1P are mediated via its specific interaction with cell-surface receptors. Recently, five mammalian G protein-coupled receptors (GPCRs), named Edg1, 3, 5, 6, and 8, have been found to be S1P receptors and their signaling properties characterized (4–14). Edg1 was shown to mediate S1P activation of MAP kinase and inhibition of adenylyl cyclase in a pertussis toxin (PTX)-dependent manner (4). Likewise, Edg8 is coupled to inhibition of adenylyl cyclase in a PTX-dependent manner (11). S1P activation of Edg3 results in calcium mobilization in a PTX-independent manner (8, 15), and inhibition of adenylyl cyclase via $G_{i/o}$ protein (15). Edg5, couples also to $G_{q/11}$ proteins (9, 15). All four S1P receptors signal in *Xenopus* oocytes, although Edg1 and Edg8 signaling were dependent on co-injection with a chimeric G_q/G_i protein (11, 16). Lysophosphatidic acid, a structurally-similar glycerol-based lysophospholipid, is a high affinity ligand for the other segment of the Edg family (Edg2, 4, and 7) (17–20).

Edg1 (endothelial differentiation gene) was cloned originally as an immediate-early response gene product (21). Since the ligand, S1P, was identified from serum via a morphologic change assay system in Edg1-overexpressing human embryonic kidney HEK293 cells, intensive studies have shown roles for S1P/Edg1 and Edg3 receptors in angiogenesis, stimulating interest in the pharmacology of these receptors (4, 22). However most of these studies have been carried out in cell culture lines and relatively little is known about the role of Edg receptors in embryonic development. In particular, localized expression of Edg1 has not been detected until fairly late in embryogenesis in mice and rats (23, 24).

These findings of bioactive lysophospholipids and the variety of their receptors evoke fundamental questions. When have bioorganisms started to use lysolipids as intercellular signaling molecules evolutionarily? When did the Edg GPCR subfamily evolve from common ancestral GPCRs and when did it diversify to its present complexity? What developmental events is the Edg receptor family involved in and is the biological function of these receptors conserved through evolution? To address these questions, we need to identify Edg receptors from simpler organisms. We have been unable to identify Edg orthologues by sequence similarity in the *Caenorhabditis elegans* or *Drosophila melanogaster* genomes, perhaps because GPCRs in these organisms are, in general, quite dissimilar to mammalian GPCRs. From the zebrafish EST database, we cloned an orthologue of the mammalian Edg1 receptor. In this report, we show that the signaling properties of zebrafish *edg1* are well-conserved through

Abbreviations used: zEdg1, zebrafish endothelial differentiation gene 1; S1P, sphingosine 1-phosphate; H2S1P, dihydro-sphingosine 1-phosphate; SPC, sphingosylphoshorylcholine; LPA, lysophosphatidic acid; PCR, polymerase chain reaction; GPCR, G protein-coupled receptor; PTX, pertussis toxin.

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Edg1-mouse ~MVSTSIPEV KALRSSVSDY GNYDIIVRHY NYTGKLNIGA EKDHGIKLTS
Edg1-rat   MVSSTSIPVV KALRSQVSDY GNYDIIVRHY NYTGKLNIGV EKDHGIKLTS
Edg1-human ~MGPTSVPLV KAHRSSVSDY VNYDIIVRHY NYTGKLNISA DKENSIKLTS
Edg1-zfish ~~~~~~~~~~ ~~~~~~~~~~ ~MDDLIARHY NFTGKFR.KV HKDPGLKADS

Edg1-mouse VVFILICCFI ILENIFVLLT IWKTKKFHRP MYYFIGNLAL SDLLAGVAYT
Edg1-rat   VVFILICCLI ILENIFVLLT IWKTKKFHRP MYYFIGNLAL SDLLAGVAYT
Edg1-human VVFILICCFI ILENIFVLLT IWKTKKFHRP MYYFIGNLAL SDLLAGVAYT
Edg1-zfish VVFIIVCCFI ILENVLVLLT IWRTKKFHKP MYYFIGNLAL SDLLAGVVYT

Edg1-mouse ANLLLSGATT YKLTPAQWFL REGSMFVALS ASVFSLLAIA IERYITMLKM
Edg1-rat   ANLLLSGATT YKLTPAQWFL REGSMFVALS ASVFSLLAIA IERYITMLKM
Edg1-human ANLLLSGATT YKLTPAQWFL REGSMFVALS ASVFSLLAIA IERYITMLKM
Edg1-zfish ANILLSGANT YKLTPTQWFF REGSMFVALA ASVFSLLAIA IERHLTMLKM

Edg1-mouse KLHNGSNSSR SFLLISACWV ISLILGGLPS MGWNCISSLS SCSTVLPLYH
Edg1-rat   KLHNGSNSSR SFLLISACWV ISLILGGLPI MGWNCISSLS SCSTVLPLYH
Edg1-human KLHNGSNNFR LFLLISACWV ISLILGGLPI MGWNCISALS SCSTVLPLYH
Edg1-zfish KLHNNGKTCR VFMLISTVWF IAAILGGLPV MGWNCIDSIN NCSTVLPLYH

Edg1-mouse KHYILFCTTV FTLLLLSIAI LYCRIYSLVR TRSRRLTFRK ..NISKGSRS
Edg1-rat   KHYILFCTTV FTLLLLSIVI LYCRIYSLVR TRSRRLTFRK ..NISKASRS
Edg1-human KHYILFCTTV FTLLLLSIVI LYCRIYSLVR TRSRRLTFRK ..NISKASRS
Edg1-zfish KAYILFCTTV FSVILMAIVI LYARIYALVR TRSRKLVFRK VANGRGSNKS

Edg1-mouse SEKSLALLKT VIIVLSVFIA CWAPLFILLL LDVGCKAKTC DILYKAEYFL
Edg1-rat   SEKSLALLKT VIIVLSVFIA CWAPLFILLL LDVGCKAKTC DILYKAEYFL
Edg1-human SE.NVALLKT VIIVLSVFIA CWAPLFILLL LDVGCKVKTC DILFRAEYFL
Edg1-zfish SEKSMALLKT VIIVLSCFIA CWAPLFILLL LDVACQTLTC SILYKAEWFL

Edg1-mouse VLAVLNSGTN PIIYTLTNKE MRRAFIRIVS CCKCPNGDSA GKFKRPIIPG
Edg1-rat   VLAVLNSGTN PIIYTLTNKE MRRAFIRIIS CCKCPNGDSA GKFKRPIIPG
Edg1-human VLAVLNSGTN PIIYTLTNKE MRRAFIRIMS CCKCPSGDSA GKFKRPIIAG
Edg1-zfish ALAVLNSAMN PLIYTLTSNE MRRAFIKMLN CGVCV..QPS GKFSRPIM.G

Edg1-mouse MEFSRSKSDN SSHPQKDDGD .NP.ETIMSS GNVNSSS* 382 aa
Edg1-rat   MEFSRSKSDN SSHPQKDDGD .NP.ETIMSS GNVNSSS* 383 aa
Edg1-human MEFSRSKSDN SSHPQKDEGD .NP.ETIMSS GNVNSSS* 381 aa
Edg1-zfish AEFSTSKSDN SSHPNKDEPE YSPRETIVSS GNITSSS* 362 aa
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FIG. 1. Alignment of the deduced amino acid sequences of the zebrafish, mouse, rat, and human Edg1 genes. The putative TMs are underlined. The zebrafish Edg1 DNA sequence has been deposited with GenBank (Accession No. AF321294).

vertebrate evolution whereas, in contrast to rodent Edg1 genes, localized zebrafish *edg1* expression starts soon after gastrulation and is restricted largely to the brain.

MATERIALS AND METHODS

Molecular cloning. During a scan of the Genbank Expressed Sequence Tags (ESTs) database using FAST_PAN (25), we found a sequence encoding a zebrafish protein that is 70% identical to human Edg1. We retrieved the underlying cDNA (clone fb98f05.y1, Research Genetics, Birmingham, AL), verified its DNA sequence and amplified the full length open reading frame of zebrafish *edg1* with a forward primer, 5′-CGG TAC CAT GGA TGA CCT AAT CG-3′ and a reverse primer, 5′-GTT CTA GAA TAG TCC CTT TAA GAA G-3′. The DNA was subcloned into the plasmid expression vector pCR3.1 (Invitrogen) and its sequence determined.

Stable expression in Rh7777 cells. Rh7777 cell monolayers were transfected with p*Zedg1*-pCR3.1 using the calcium phosphate precipitate method and clonal populations expressing the neomycin phosphotransferase gene were selected by addition of geneticin (G418) to the culture media. The Rh7777 cells were grown in monolayers at 37°C in a 5% CO_2/95% air atmosphere in a growth media consisting of 90% MEM, 10% fetal bovine serum, 2 mM glutamine, and 1 mM sodium pyruvate.

Measurement of cAMP accumulation. Assay of adenylyl cyclase activity (i.e., cAMP accumulation) was performed as described previously by us (11). These assays were conducted on populations of 5×10^4 cells stimulated with 1 μM forskolin in the presence of the phosphodiesterase inhibitor isomethylbutylxanthine. cAMP was measured by automated radioimmunoassay.

GTPγ[^{35}S] binding. Briefly, 25 μg of membranes from *edg1* stably-transfected Rh7777 cells were incubated in 1.0 ml GTP-binding buffer (in mM: HEPES 50, NaCl 100, $MgCl_2$ 5, pH 7.5) containing 25 μg saponin, 10 μM GDP, 0.1 nM GTPγ[^{35}S] (1200 Ci/mmol), and indicated lipid for 30 min at 30°C. Membrane bound radionuclide was collected using a Brandel Cell Harvester (Gaithersburg, MD).

Sources of materials. Rh7777 cells (CRL 1601) were from the American Type Culture Collection (Manassas, VA), human Edg1 cDNA was a gift from Dr. Timothy Hla (University of Connecticut), 1-oleoyl LPA was purchased from Avanti Polar Lipids (Alabaster, AL), S1P from Biomol (Plymouth Meeting, PA), GTPγ[^{35}S] from New England Nuclear (Boston, MA), geneticin, cell culture media and sera from GibcoBRL Life Technologies (Bethesda, MD), oligonucleotides from Operon Technologies (Alabama, CA), expression plasmids were from Invitrogen (La Jolla, CA), other chemicals were from Sigma (St. Louis, MO).

In situ hybridization and histology. Antisense and control sense digoxigenin-UTP-labeled *edg1* RNA probes were transcribed *in vitro* from *XbaI* (or *KpnI* for sense) linearized p*Zedg1*-pCR3.1 (from clones in antisense and sense orientation) by T7 polymerase, following Harland (26) except that probes were not alkali-hydrolyzed. *In situ* hybridization was performed as described in Ungar *et al.* (27), except that embryos were permeabilized for 1–6 min, depending on stage, in 2.5 μg/ml Proteinase K in phosphate buffered saline (PBS) containing 0.1% Tween 20, and postfixed in 4% formaldehyde in PBS. Hybridization overnight and washing at 60 or 64°C yielded similar results.

For sectioning, labeled embryos were fixed overnight in 4% formaldehyde in PBS, dehydrated through methanol, ethanol, 1:1 ethanol:xylenes, xylenes, and 1:1 xylenes:paraplast plus, then infiltrated overnight in paraplast plus, incubated twice in paraplast plus, and embedded. 10 μm sections were cut.

RESULTS

Cloning of Zebrafish edg1

We found in the Expressed Sequence Tag (EST) database a sequence encoding a zebrafish protein that is

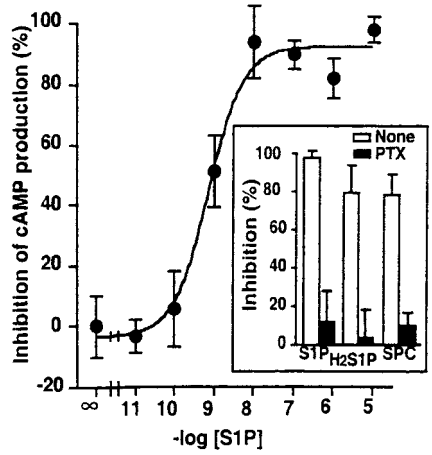

FIG. 2. Dose–response curve of inhibition of forskolin-induced cAMP production in response to S1P. Insert shows effect of PTX on the inhibitory actions of lysosphingolipids. Closed blocks are PTX treated (100 ng, 24 h). Open boxes are represent untreated cells.

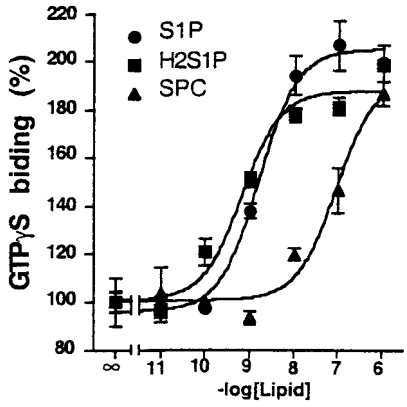

FIG. 3. Dose–response curves of GTPγ[35S] binding to zEdg1-Rh7777 cell membranes in response to S1P, dihydro-S1P and SPC.

about 70% identical to human Edg1. We amplified the full-length translational open reading frame of zebrafish *edg1* and determined nucleotide sequence. The deduced amino acid sequence of the zebrafish Edg receptor is shown in Fig. 1, along with an alignment to Edg protein sequences from other species. The protein sequence of zebrafish DNA shares 71% identical amino acids with human Edg1, is 47–54% identical to Edg3, 5, and 8 but only 33–37% identical to Edg2, 4, and 7. Because the zebrafish gene is clearly most similar to Edg1, we have named it zebrafish *edg1*.

Zebrafish edg1 Encodes a Functional G Protein-Coupled S1P Receptor

From its sequence similarity to mammalian Edg1, we hypothesized that zebrafish *edg1* encodes a receptor for S1P. To test whether the recombinant zEdg1 could transduce a cellular signal in response to S1P, we transfected zebrafish *edg1* stably into rat hepatoma Rh7777 cells—a cell line that shows only small responses to applied S1P (11). Rh7777 cells stably expressing the zebrafish *edg1* receptor were assayed for inhibition of forskolin-stimulated cAMP produc-

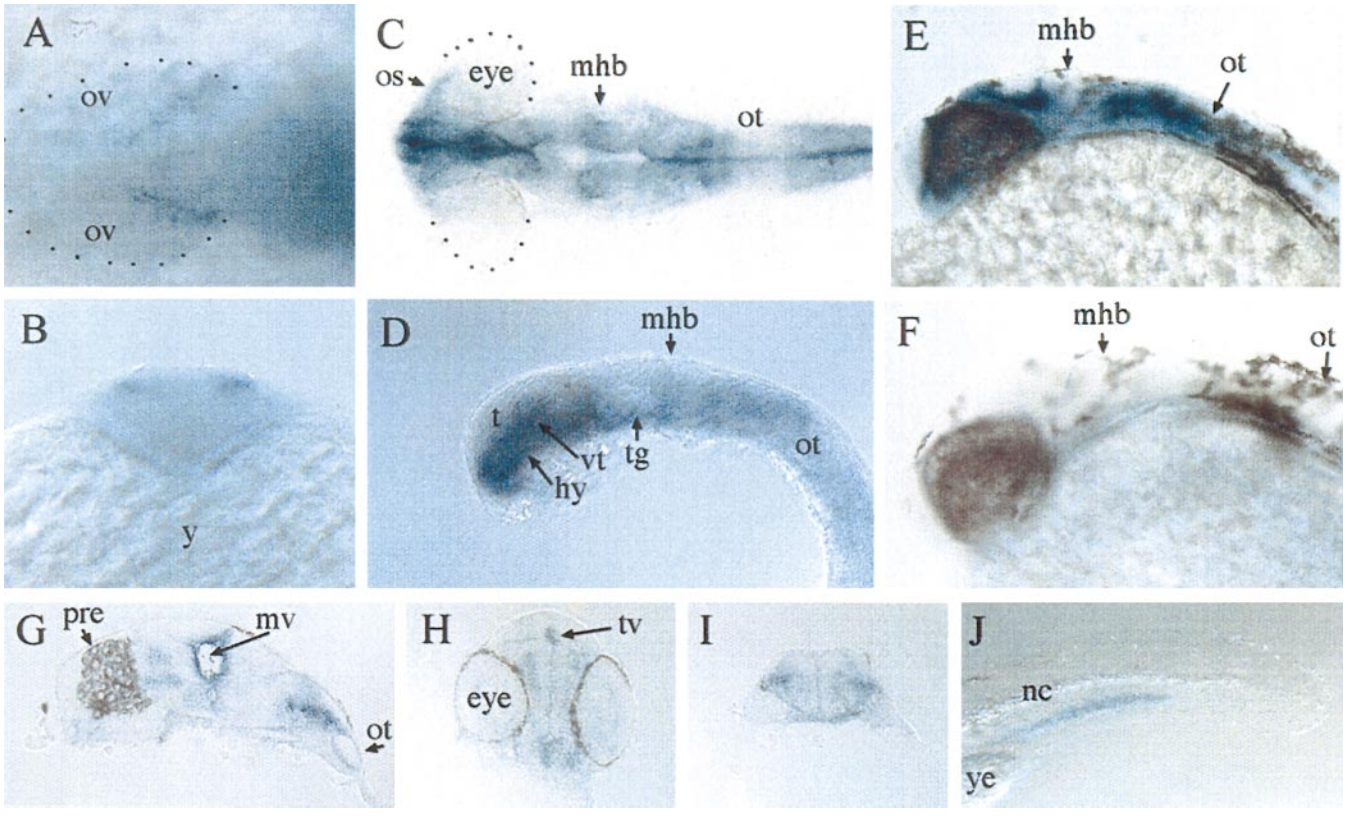

FIG. 4. Edg1 is transcribed during embryogenesis, primarily in the brain. Embryos were hybridized with edg1 antisense probe, except for the embryo shown in F, which was hybridized with edg1 sense probe as a control. (A) Animal pole view of a 4 somite stage embryo. Dots were added to outline the evaginating optic vesicles. (B) Optical cross section through the forebrain of a 4 somite stage embryo. (C) Dorsal view of the head of a 24 h embryo. The dots outline the eyes. (D) Lateral view of the head of a 24 h embryo. (E) Lateral view of a 34 h embryo. (F) Lateral view of a 34 h embryo hybridized with control sense probe. (G) Parasaggital section through the head of a 34 h embryo. (H) Cross section through the forebrain of a 34 h embryo. (I) Cross section through the anterior hindbrain of a 34 h embryo. (J) Tail of a 34 h embryo. Abbreviations: hy, hypothalamus; mv, mesencephalic ventricle; mhb, midbrain-hindbrain boundary; nc, notochord; os, optic stalk; ot, otic vesicle; ov, optic vesicle; pre, pigmented retinal epithelium; tg, tegmentum; tv, third (diencephalic) ventricle; vt, ventral thalamus; y, yolk; ye, yolk extension.

tion. S1P at concentrations as low as 100 pM, but not LPA at concentration as high as 10 μM, was able to inhibit intracellular cAMP production significantly in the receptor-transfected cells. The inhibitory effect of S1P was found to be dose-dependent with an IC_{50} value of 1 nM (Fig. 2). Furthermore, dihydro-S1P and sphingosylphosphorylcholine (SPC), which are structurally-related agonists at mammalian S1P receptors, show inhibition of cAMP accumulation. Other structurally related lysophospholipids, e.g. lysophosphatidylserine, lysophosphatidylcholine, lysophosphatidylinositol, lysophosphatidylethanolamine and lysophosphatidylglycerol were not active on these cells (data not shown). Therefore, the ligand specificity of zebrafish *edg1* appears to be similar to mammalian S1P Edg receptors.

G proteins were implicated in the S1P-zebrafish *edg1* signaling using two assays. In the first assay, Rh7777 cells stably transfected with *edg1* were treated with pertussis toxin (PTX), which ADP ribosylates $G_{i/o}$ proteins specifically and inhibits them. Treatment with 100 ng of PTX for 24 h blocked entirely the ability of S1P, dihydro-S1P, and SPC to inhibit cAMP accumulation (Fig. 2, inset). This result suggests the involvement of $G_{i/o}$ proteins in edg1 signaling. Secondly, a GTPγS binding assay was performed with membranes prepared from zebrafish *edg1*-Rh7777 cells. S1P, dihydro-S1P and SPC increased the binding in a dose-dependent manner and the EC_{50} values were about 1.2 nM, 0.8 nM, and 100 nM respectively (Fig. 3).

Embryonic Expression of edg1

To determine when and where edg1 might function in development, we examined *edg1* expression in zebrafish embryos by *in situ* hybridization. We did not detect *edg1* expression in blastula or gastrula stage embryos (data not shown). At 4–5 somites (about 2 h after gastrulation), *edg1* transcript is found in a narrow row of cells in the diencephalon just posterior to the optic vesicles (Fig. 4A). Only the most dorsal neural cells are expressing *edg1* at this stage, as shown in the optical section in Fig. 4B. Weak expression is also visible in the hindbrain at this stage (data not shown). We also detected faint staining in the tailbud (data not shown), which was not observed at later somitogenesis stages.

By 14 somites, *edg1* expression has become more widespread in the CNS and the pattern of *edg1* expression appears similar at 24 h (Figs. 4C and 4D). In the forebrain, *edg1* is expressed in most of the diencephalon, with strongest expression in the hypothalamus and in the optic stalks. *Edg1* expression is low or absent in the eyes and in the telencephalon. *Edg1* is expressed at the forebrain-midbrain and midbrain-hindbrain boundaries. In the hindbrain, *edg1* expression is most intense in rhombomere 4, and largely absent from the posterior hindbrain. *Edg1* is also expressed in the anterior spinal cord.

By 34 h, *edg1* staining is prominent in cells near the ventricles of the brain (Figs. 4E and 4G). This staining was not observed in control embryos hybridized with a sense *edg1* probe (Fig. 4F). There are also several groups of cells expressing *edg1* in the diencephalon, just medial to the eyes, seen in the parasaggital section in Fig. 4G, and in the cross section in Fig. 4H. In the hindbrain, *edg1* expression extends from rhombomeres 1 through 4 (Figs. 4E and 4G). This hindbrain expression is only present lateral to the midline and is most intense at an intermediate dorso-ventral position (Fig. 4G). Also at this stage, *edg1* staining is present ventrally in the tail (Fig. 4H).

DISCUSSION

In this study, we show four findings, (i) molecular cloning of the zebrafish *edg1* receptor, (ii) functional activation of the receptor with S1P in intact mammalian cells by inhibition of adenylyl cyclase, (iii) G protein-couplings of the receptor by GTPγS binding in membrane preparations and PTX treatment, and (iv) expression of *edg1* in the developing zebrafish brain.

The inhibitory effect of S1P on adenylyl cyclases are a well-characterized response of mammalian S1P receptors, Edg1, 3, and 8 (6, 11, 15). The effects of dihydro-S1P and SPC on zebrafish *edg1* are not different from those on human Edg1 (data not shown). Dihydro-S1P is equipotent and SPC is a lower potency, partial agonist at both Edg receptors, suggesting that ligand specificity of Edg1 receptors may be highly conserved throughout evolution. Because the medicinal chemistry of this ligand is not well developed, we could not compare a variety of agonists and antagonists. Our results furthermore imply a conservation of the functional characteristics of the S1P in fish and mammalian species in terms of PTX-sensitive G-protein activation and provide a clue for understanding of the molecular evolution of the Edg receptor cluster and physiological function in vertebrates of lysolipid phosphoric acids.

Eight different receptor subtypes for lysolipid phosphoric acids have been cloned in mammals. Recently, puffer fish *edg3* was cloned and found to be 65% identical to human Edg3 (28), but functional couplings and expression patterns of the receptor were not characterized. To consider the evolutionary events that have generated the various lysophospholipid phosphoric acid receptor subtypes, though, we need to wait for more genes cloned from teleost fishes and other simpler vertebrates. Our data indicate that the S1P receptor was present early in the evolution of vertebrates and that pharmacological and functional properties of this receptor have been conserved over a period of 400

million years, which is the evolutionary distance between teleost fish and modern mammals.

We have shown that zebrafish *edg1* expression appears in discrete regions of the brain shortly after the completion of gastrulation. At this time, the initial regionalization of the brain has already occurred, but in zebrafish, *edg1* appears early enough to play any of a variety of roles in subsequent developmental events. Based on studies of the activity of S1P in cell culture systems, edg1 may function in the developing CNS in cell proliferation, apoptosis, morphogenesis, adhesion, or axon guidance (1–4).

This early, localized neural expression in zebrafish contrasts with reports from rodent *edg1* genes. In mouse embryos, *edg1* is expressed only diffusely and at low levels prior to E15.5, when higher level expression was observed throughout the entire brain and in the liver, lung, heart, and developing skeleton, becoming particularly abundant in the newborn hippocampus (23). In the rat, *edg1* expression appears to be similar to that in the mouse; in particular, transcript is first detected at E15 (24).

Edg1 was first identified as an endothelial differentiation gene in mammals. In contrast to mammalian findings, we did not detect much expression of *edg1* outside of the CNS in zebrafish embryos. It is possible that a second zebrafish *edg1* orthologue exists, since a whole-genome duplication occurred sometime after the divergence of the zebrafish and mammalian lineages and the expression domains of the two duplicated zebrafish orthologues frequently have diverged from one another (reviewed in (29)).

Our work describing the cloning, pharmacology, and developmental expression of zebrafish *edg1* should be a useful basis to explore the role of S1P and its receptors in vertebrate development. Furthermore, our finding of the *edg1* receptor may help to develop the medicinal chemistry of S1P and to find receptor subtype selective agonists and antagonists, because evolutionarily conserved residues of Edg1 could elucidate the interaction sites of the receptor with S1P.

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