Characterization of a Novel Sphingosine 1-Phosphate Receptor, Edg-8*

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Dong-Soon Im‡, Christopher E. Heise§§, Nicolas Ancellin¶, Brian F. O'Dowd¶, Gan-ju Shei**, Robert P. Heavens‡‡, Michael R. Rigby‡‡, Timothy Ha¶¶, Suzanne Mandala**, George McAllister**, Susan R. Georgei, and Kevin R. Lyncii

From the ¶Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908, the §Center for Vascular Biology and Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06030, the ¶¶Center for Addiction and Mental Health and University of Toronto, Toronto, Ontario M5S 2S1, Canada, the §§Department of Infectious Disease, Merck Research Laboratories, Rahway, New Jersey 07065, and ¶¶¶Merck, Sharpe & Dohme, Neuroscience Research Center, Harlow CM20 2QR, United Kingdom

Three G protein-coupled receptors (Edg-1, Edg-3, and Edg-5) for the lysolipid phosphoric acid mediator sphingosine 1-phosphate have been described by molecular cloning. Using a similar sequence that we found in the expressed sequence tag data base, we cloned and characterized a novel high affinity, rat brain sphingosine 1-phosphate receptor, Edg-8. When HEK293T cells were co-transfected with Edg-8 and G protein DNAs, prepared membranes showed sphingosine 1-phosphate-dependent increases in [³²P]guanosine 5'-O-thiotriphosphate binding with an EC₅₀ of 90 nM. 

Sphingosine 1-phosphate (S1P)¹ is a potent, extracellular lysolipid phosphoric acid mediator that is released, for example, during platelet activation (1). S1P elicits a wide variety of responses by cells; prominent among these are cell proliferation (2-4) and anti-apoptosis (5, 6) as well as a wide variety of other effects. S1P and the structurally related lysolipid mediator, lysosphosphatidic acid (LPA), are recognized now to signal cells through a set of G protein coupled receptors known colloquially as the “Edg” receptors. Discovered initially as “orphan” receptors (7, 8), three members of the group, Edg-1, Edg-5, and Edg-3, have been shown to be S1P receptors. For example, Edg-1 mediates S1P activation of mitogen-activated protein kinase and inhibition of adenylyl cyclase in a pertussis toxin-dependent manner (9, 10). S1P activation of Edg-3 results in calcium mobilization in a pertussis toxin-dependent manner (11), while others found that this receptor coupled also, for example, to inhibition of adenylyl cyclase via G₁₁α protein (12). Likewise, several groups have shown that a third S1P receptor, Edg-5, couples also to G₁₁α proteins (13, 14). All three S1P receptors signal in Xenopus oocytes, although Edg-1 signaling was dependent on co-injection with a chimeric G₁₆α protein (15). These data suggest that the three known S1P receptors interact with different signal transduction pathways. Indeed recent biochemical evidence supports this notion, Edg-1 interacts with G₁α, whereas Edg-3 and Edg-5 interact with G₁₁α and G₁₆α as well as G₁α in a ligand-dependent manner (14). Three of the remaining four Edg family proteins (Edg-2, -4, and -7), which form a cluster as judged by amino acid sequence similarity, are LPA receptors, while a fourth member (Edg-6) remains an orphan receptor. In this paper, we report the existence of another S1P receptor, Edg-8, which we found first as a deposition in the expressed sequence tag data base and later cloned from rat brain. Edg-8 binds S1P with high affinity, couples to G₁₁α proteins, and is localized prominently to white matter in the adult rat brain.

EXPERIMENTAL PROCEDURES

Cloning of Rat Edg-8 cDNA—We found a partial rat nucleotide sequence (GenBank™ accession number A1317881) similar to Edg-1, Edg-5, and Edg-3 during a routine search of updates to the GenBank™ database of expressed sequence tags (ESTs) using the FAST_PAN program (16). We used this DNA sequence to design oligonucleotide primers that were in turn used to amplify a fragment of rat brain cDNA.

¹ The abbreviations used are: S1P, sphingosine 1-phosphate; dihydro-S1P, sphinganine 1-phosphate; LPA, lysosphosphatidic acid; Edg, endothelial differentiation gene; SPC, sphingosylphosphorylcholine; GPCR, G protein-coupled receptor; EST, expressed sequence tag; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; GTPγS, guanosine 5’-O-thiotriphosphate.

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After verifying that the partial cDNA had the same nucleotide sequence as the EST DNA, the former was used to screen a rat brain cDNA library as we have reported previously (17). Three independent cDNA clones were identified and their nucleotide sequences were determined. From one of these cDNAs was subcloned into the plasmid expression vector pcDNA3 for further analysis.

**Transient Expression in HEK293T Cells—**Edg-8 DNA was mixed with an equal amount of DNA encoding a mutated (C351F) rat G_i2 protein as well as DNA encoding cow β_i and γ_2 proteins and used to transfect monolayers of HEK293T cells using the calcium phosphate precipitate method (18). After about 8 h, cells were harvested, membranes were prepared, aliquoted, and stored at –70°C until use.

\[^{35}S\]GTP-γ-S Binding—Briefly, 25 μg of membranes from Edg-8 expressing HEK293T cells were incubated in 1.0 ml of GTP-binding buffer (in mM: HEPES, 50; NaCl, 100; MgCl_2, 5.0; pH 7.5, containing 25 μg of saponin, 10 μM GTP, 0.1 μM \[^{35}S\]GTP-γ-S (1200 Ci/mmol), and test lipid. After incubating for 30 min at 30°C, bound radionuclide was separated from free by filtration through Whatman GF/C paper using a Brandel Cell Harvester (Gaithersburg, MD).

**Stable Expression in Rh7777 Cells—**Rat hepatoma Rh7777 cell monolayers were transfected with Edg-8-pcDNA3 DNA using the CaCl_2 procedure from Specialty Media (Lavellette, NJ). Cells were seeded at 105 cells stimulated with 1 mM Tris/Cl, pH 7.5, 200 mM NaCl, 30 mM NaF, 4 mM deoxyguanosine, protease inhibitor mixture and stored frozen at 70°C until use.

**Radioligand Binding**—For the binding assay, \[^{3}P\]sphingosine 1-phosphate was sonicated with fatty acid-free BSA and added to membranes in 200 μl in 96-well plates with assay concentrations of 1 nM \[^{3}P\]sphingosine 1-phosphate (20,000 dpm, 4 μg/ml BSA, 20 μM Tris/Cl, pH 7.5, 100 mM NaCl, 15 mM NaF, 2 mM deoxyguanosine, protease inhibitor mixture and stored frozen at 70°C until use.

**RNA Analysis, Northern Blotting—**RNA extraction, Northern blotting, and hybridization of radiolabeled Edg-8 DNA were as described previously by us (17).

**RESULTS**

**Fig. 1A** shows the DNA sequence and deduced amino acid sequence of rat Edg-8. This sequence is from a single cDNA, but is the same sequence that we found in three independent cDNAs and the EST cDNA in regions of overlap. Therefore the sequence we report most likely represents the coding region of the rat brain Edg-8 mRNA population. The longest cDNA that we isolated contained about 80 nucleotides upstream of the putative initiation codon, but no in-frame termination codon was found in this region. Although we could not be certain that we have isolated the full translational region from our data alone, a recent report (22) of the cloning of the same sequence as an orphan receptor-related nrg-1, suggested a discussion of the degree of identity, a longer cDNA that does contain an in-frame termination codon at position –102. Using the BLAST (23) and FASTA (24) search algorithms, we found only the single EST (GenBank™ accession number AI317881) as a record of a rat Edg-8 mRNA sequence.
family members, we found another deposition closely related to rat Edg-8. This was a human sequence in the HTGS (high through-put genome sequence) division (GenBank™ accession number AC011461) that contained an intron-less translational open reading frame encoding what is probably the human ortholog of Edg-8. The human amino acid sequence conceptualized from this deposition is 87% identical to rat Edg-8 and thus might be the human ortholog of rat Edg-8.

The conceptualized rat Edg-8 protein (400 amino acids, 42,301 daltons) is 42–49% identical to the S1P receptors Edg-1, -3, and -5 and 33–36% identical to the LPA receptors Edg-2, -4, and -7. Furthermore, Edg-8 is 45% identical to the orphan receptor, Edg-6 (25), and 34–35% identical to the orphan GPCR cluster GPR3/6/12, but less than 28% identical to any other GPCR amino acid sequence. An alignment of rat Edg-8 with the three known S1P receptors is presented as Fig. 1B.

Hydropathy analysis of Edg-8 (not shown) suggests the heptahelix motif assumed to be common to GPCRs and the protein has the conserved amino acid motifs expected of a rhodopsin-like (Family A) GPCR.

Given the close similarity of rat Edg-8 to known S1P receptors, we tested S1P as a potential ligand in a variety of assays. To accomplish this, we required systems wherein the ubiquitous endogenous S1P responses are small or negligible. One of these is a [35S]GTPγS binding assay using HEK293T cell membranes from cells transfected with Edg-8 DNA. Although this cell line responds to S1P2 and expresses S1P receptors (26), membranes prepared from these cells exhibit very little [35S]GTPγS binding in response to S1P. In contrast, co-transfection of these cells with DNAs encoding various Edg family 2 D.-S. Im and K. R. Lynch, unpublished data.

FIG. 1 — continued

Table 2. [35S]GTPγS binding to HEK293T cell membranes in response to S1P, dihydro-S1P (H,S1P), and SPC. The absolute values for [35S]GTPγS binding are: basal (no drug) 2887 ± 11 dpm and maximum, 4531 ± 26 dpm. Each data point represents mean of six determinations ± S.E.

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receptors, and G proteins results in robust \[^{35}\text{S}][\text{GTP}]\gamma\text{S}

\text{binding in response to the appropriate lysolipid phosphate ligand.}^3\text{ For example, Fig. 2 shows a dose-response curve for S1P-}\[^{35}\text{S}][\text{GTP}]\gamma\text{S}\text{ binding to HEK293T membranes from cultures co-transfected with DNAs encoding rat Edg-8, rat G}_{\omega}\text{C351F protein, and cow }\beta_1\gamma_2 \text{ G proteins. The EC}_{50}\text{ for S1P or dihydro-S1P in this assay was 90 nM; and while LPA and other glycolerol-based lyso phospholipids at concentrations up to 10 }\mu\text{M did not stimulate binding (not shown), sphingosylphosphorylcholine (SPC) did stimulate }[^{35}\text{S}][\text{GTP}]\gamma\text{S binding, albeit with reduced potency and efficacy. Partial agonism by SPC was reported also with Edg-1, Edg-3, and Edg-5 receptors expressed in Xenopus oocytes (15).}

To examine the signaling properties of recombinant Edg-8, we introduced this DNA into Rh7777 rat hepatoma cells by transfection and selected for Geneticin-resistant clonal populations. Rh7777 cells were used because they exhibit only modest endogenous responses to S1P (27). After transfection with Edg-8 DNA, however, S1P or dihydro-S1P treatment resulted in about 80% inhibition of forskolin-driven rises in adenylyl cyclase (Fig. 3), and the sensitivity to S1P increased about 2 log orders as compared with the endogenous response (IC\text{50} 120 nM). S1P, dihydro-S1P, and SPC all stimulated the EC\text{50} value calculated from this curve was 5.4 nM. Light emission was recorded for 120 s. Results are mean ± S.E. derived from seven different experiments.

Furthermore, sphingosine, C\text{2-ceramide, ceramide 1-phos-}}
phate, and sphingomyelin were unable to stimulate Edg-8/Gi/o-expressing oocytes (at concentrations up to 1 μM), while SPC was a low potency partial agonist (data not shown). In this assay, we found that a chimeric Gαq protein was functional also (data not shown). As with our other expression systems, dihydro-S1P was indistinguishable from S1P (data not shown). The requirement for co-expression of the chimeric G protein supports the conclusion that Edg-8 prefers coupling to a G protein of the Gi/o type.

To measure the affinity of S1P for Edg-8, we prepared 33P-labeled S1P and used this compound in a receptor binding assay. As shown in Fig. 5, radiolabeled S1P was displaced by S1P, dihydro-S1P, and SPC from membranes prepared from Edg-8 DNA-transfected HEK293T or Rh7777 cells. The binding constant (KD) for S1P calculated from the displacement curve was 2 nM, and membranes from both cell types showed high affinity binding. As predicted by the aforementioned studies of receptor function (30), dihydro-S1P was equipotent to S1P, while SPC was relatively ineffective in displacing the radioligand.

We investigated the expression pattern of the rat Edg-8 gene in several tissues. Northern blot analyses showed a prominent band migrating at 2.2 kilobases in extracts of various rat tissues, including throughout the brain and spleen (Fig. 6). To explore rat brain Edg-8 gene expression at higher resolution, we performed in situ hybridization with adult rat brain sections. This analysis (see Fig. 7) revealed a striking pattern, i.e. Edg-8 mRNA was expressed predominantly within white matter tracts of the rat brain. Corpus callosum, optic nerve, olfactory tract, anterior commissure, internal and external capsules, fimbria of the hippocampus, mamillary tract, stria medullaris, and white matter of the cerebellum and brain stem all showed specific signal. Within the striatum itself white matter fascicles could be identified.

**DISCUSSION**

Sphingosine 1-phosphate and the structurally similar lysosphosphatidic acid mediator, lysophosphatidic acid, have been long suspected to signal cells at least in part through G protein-coupled receptors. Given the plethora of responses that these mediators elicit from cells and tissues, it is not surprising that there exists multiple receptor subtypes for both lipids. In the present study we have used two mammalian cell lines and frog oocytes to demonstrate unequivocally that Edg-8 is a fourth, high affinity sphingosine 1-phosphate receptor. Edg-8 is similar to Edg-1 in that both S1P receptors are predominantly Gαi-linked and are unable to couple to the Gqα pathway. Rat tissue expression data, however, indicate that Edg-8 is not expressed nearly as widely as Edg-1. Like the other S1P receptors, Edg-8 does not discriminate between S1P and its reduced
form, dihydro-S1P (sphinganine 1-phosphate). The addition of a choline head group (SPC) results in a weak, partial agonist at this and other S1P receptors, and lysolipids that are not sphingosine-based are not active in our assays at concentrations up to 10 μM.

The existence of Edg-8 was not predicted from existing knowledge of S1P biology, the responses of cells and tissues to this lipid mediator could apparently be explained by the three known S1P receptors. However, there exists an interesting correspondence between S1P and LPA signaling systems in many cell types. Interestingly, the LPA receptor, Edg-2, is a Gα protein-linked receptor that is expressed predominantly by oligodendrocytes in rat (28) and mouse (29) brains. Perhaps the most intriguing aspect of our study is that Edg-8 is similar to Edg-2 in that expression in the CNS of both receptors is restricted to white matter. Although the present study does not allow discrimination between Edg-8 expression in oligodendrocytes versus fibrous astrocytes (or microglia or low level expression in neurons), the pattern of Edg-8 gene expression is perhaps yet another example of a parallel between the S1P and LPA signaling systems in neurons (as judged by Northern blotting, see Fig. 6). The nrg-1 gene was mapped to rat chromosome 8 where it is found tightly linked to rat Edg-5 (also known as H218). This linkage might be preserved in the human genome as both the Edg-8 and Edg-5 transatlational open reading frames are found in bacterial artificial chromosomes from human chromosome 19.

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