J. Biochem. **131**, 767–771 (2002)

The Edg Family G Protein–Coupled Receptors for Lysophospholipids: Their Signaling Properties and Biological Activities[1]

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Received April 11, 2002; accepted April 17, 2002

Sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are blood-borne lysophospholipids with a wide spectrum of biological activities, which include stimulation of cell growth, prevention of apoptosis, regulation of actin cytoskeleton, and modulation of cell shape, cell migration, and invasion. Activated platelets appear to be a major source of both S1P and LPA in blood. Despite the diversity of their biosynthetic origins, they are considered to share substantial structural similarity. Indeed, recent investigation has revealed that S1P and LPA act *via* a single family of G protein–coupled receptors designated as Edg. Thus, the Edg isoforms, Edg1 (also called $S1P_1$), Edg5 ($S1P_2$), Edg3 ($S1P_3$), Edg6 ($S1P_4$), and Edg8 ($S1P_5$), are specific receptors for S1P (and SPC with a lower affinity), whereas Edg2 (LPA_1), Edg4 (LPA_2), and Edg7 (LPA_3) serve as receptors specific for LPA. Each receptor isoform displays a unique tissue expression pattern and coupling to a distinct set of heterotrimeric G proteins, leading to the activation of an isoform-specific panel of multiple intracellular signaling pathways. Recent studies on knockout mice have unveiled non-redundant Edg receptor functions that are essential for normal development and vascular maturation. In addition, the Edg lysophospholipid signaling system may play a role in modulating cell motility under such pathological conditions as inflammation, tumor cell dissemination and vascular remodeling.

Key words: endothelial differentiation gene, lysophosphatidic acid, lysophospholipid, sphingosine-1-phosphate.

Lysophospholipids constitute a group of important lipid mediators. LPA, a glycerolysophospholipid, was the first to be recognized as a major lipid mediator in serum that exerts growth factor–like activities at submicromolar concentrations (*1*). These include stimulation of mitogenesis, protection from apoptosis, induction of actin cytoskeletal reorganization and cell shape changes, and stimulation of tumor cell invasion (*2, 3*). More recently, the lysosphingolipid S1P has been found to share some biological activities with LPA, although unlike LPA, it inhibits migration and invasion of certain types of tumor cells (*4–9*). Despite the difference in the backbone structures of these two lysophospholipids, they are considered to share some similarity in their conformation (*2–5*). Biosynthesis of the two lysophospholipid mediators is described in detail in several previous publications (*2–4, 7*). S1P and LPA are both present in blood plasma, largely in albumin- and lipoprotein-bound forms, in concentration ranges of at least 10^{-7} M, and in serum at higher concentrations. One of the major sources of both S1P and LPA in serum appears to be activated platelets (*10, 11*). In addition, mast cells and monocytes are capable of producing S1P, and adipocytes of producing LPA, in response to extracellular stimuli (*7, 12*). Thus, both S1P and LPA could act as circulating, as well as locally produced, paracrine mediators. It is also suggested that the biosynthetic enzymes for the lysophospholipids are released into extracellular space, where they produce S1P and LPA from lipid precursors such as those contained in lipoproteins (*13, 14*).

I. Identification of receptors for LPA and S1P

More than a decade ago, the mitogenic action of LPA on fibroblasts was demonstrated to be sensitive to pertussis toxin (PTX), suggesting the existence of a cognate G protein-coupled receptor (GPCR) for LPA. Similarly, S1P was suggested to exert its effects *via* cell surface GPCR at least in some types of cells. In a different line of investigation, several laboratories including ours have cloned novel putative GPCRs from a variety of cDNA libraries. These include (in a chronological order): Edg1, which stands for endothelial differentiation gene as was originally cloned as a phorbol ester-inducible immediate early gene in vascular endothelial cells (*15*); AGR16/H218/Edg5, which we cloned by degenerate PCR strategies from an aortic smooth muscle and brain cDNA library (*16*); Edg2/vzg-1, which was cloned

[1] This work was supported by grants from the Ministry of Education, Science and Culture of Japan, the Japan Society for the Promotion of Science Research for the Future Program, and the Hoh-Ansha Foundation.

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Abbreviations: Edg, endothelial differentiation gene; S1P, sphingosine-1-phosphate; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; ERK, extracellular signal–regulated kinase; PTX, pertussis toxin; PLC, phospholipase C; PI, phosphatidylinositol; CHO, Chinese hamster ovary; IGF-I, insulin-like growth factor–I; AC, adenylate cyclase; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein.

from a neuronal cell cDNA library (*17*); and Edg3 (*18*). They share substantial homology in predicted amino acid sequences and thus were considered to constitute a family of "orphan" GPCRs, of which the ligand remained undetermined. In 1996, Chun and his colleagues (*19*) demonstrated that overexpression in cultured neuronal cells of Edg2/vzg-1 conferred enhanced morphological responses to LPA, providing the first evidence that a member of the Edg family represented a functional receptor for a lysophospholipid mediator. Edg2/vzg-1 is 36% homologous in predicted amino acid sequences with Edg1. In 1998 several groups including ours independently identified Edg1 as a receptor for S1P (*20–22*). We and other groups have also identified AGR16/Edg5 and Edg3 as receptors for S1P (*23–26*). Subsequently, additional members of the Edg family were cloned and identified as receptors specific for either S1P (Edg6 and Edg8) or LPA (Edg 4 and Edg7) (*5, 14, 27–30*). Thus, the Edg family GPCRs consists of two subfamilies: the S1P receptor subfamily, which comprises Edg1, Edg3, Edg5, Edg6, and Edg8; and the LPA receptor subfamily, which comprises Edg2, Edg4, and Edg7 (Table I) (*5, 8, 9, 14, 26, 27, 30*). Comparison of the amino acid sequences reveals 46–54% identity among the S1P receptor isoforms and among the LPA receptor isoforms, and a lower 32–36% identity between the two receptor subfamilies, confirming that the Edg receptors are both functionally and structurally separated into the two subfamilies. Earlier studies claimed that S1P acts as an intracellular second messenger (*7, 12*). It is now widely accepted that most, if not all, of the effects of S1P and LPA are exerted from outside of cells *via* the Edg member receptors. In addition to the Edg LPA receptors, it was reported that a GPCR termed PSP24, which has a low sequence homology to the Edg family members, serves as a receptor for LPA, although its functional properties are not fully understood (*31*). It has also been reported recently that SPC, which was recognized as a lower affinity agonist for the S1P receptor isoforms of the Edg family, serves as an agonist for other orphan GPCRs, OGR1 and GPR4 (*32, 33*).

Among the LPA receptors, Edg2 is widely expressed in almost all tissues, with the highest abundance in brain and heart and the lowest abundance in liver and peripheral blood leukocytes (*30*). The distribution of Edg4 transcript is totally different; it is readily detectable in testis, pancreas, prostate, spleen and thymus, but is almost undetectable in brain, heart, placenta, and digestive tract, where Edg2 is abundantly expressed (*30*). Edg7 exhibits a more restricted expression pattern, being confined to testis, prostate, pancreas, heart and lung (*29*). Transcripts of the S1P receptors Edg1, Edg3, and Edg5 are widely distributed in almost all organs and tissues of adult animals and some embryonic tissues examined (*27, 28*). In contrast, expression of transcript of Edg6 is confined to lymphoid tissues including spleen, lymph node and thymus, and that of Edg8 to the central nervous system (*28, 30*).

II. Signaling characteristics of the Edg receptors

Heptahelical GPCRs generally transduce signals *via* heterotrimeric G proteins, although transmembrane signalings *via* direct interaction of an intracellular domain of a receptor with an effector molecule are demonstrated in some of GPCR receptors. Several laboratories, including ours, have extensively studied the transmembrane signaling mechanisms of the cloned Edg receptors by using mammalian and non-mammalian heterologous expression systems, using cells with low or no background responsiveness to either LPA or S1P (*5, 7–9, 20–22, 25–28, 30*). These include Chinese hamster ovary (CHO) cells, B103 neuroblastoma cells, RH7777 hepatoma cells and Sf9 insect cells. Table I summarizes the results of these studies.

Edg2 couples to mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) in a PTX-sensitive manner, indicating that Gi/o mediates this response. Gi/o also mediates inhibition of adenylate cyclase upon stimulation of Edg2. Besides the Gi/o-mediated pathway, Edg2 is coupled to phospholipase C (PLC), largely by a PTX-insensitive mechanism that probably involves Gq. The third pathway activated by Edg2 is a Rho-dependent signaling cascade that leads to stress fiber formation in fibroblasts and cell rounding and neurite retraction in neuronal cells, which was evidenced by the fact that these responses were inhibited by botulinum C3 toxin, which ADP-ribosylates and inactivates Rho. This Rho-dependent pathway is probably mediated *via* $G_{12/13}$. Edg4 and Edg7 show signaling properties similar to those observed for Edg2, but unlike Edg2 and Edg4, Edg7 is not coupled to the Rho pathway. These observations in heterologous expression systems are quite consistent with those reported earlier in various cell types. Indeed, it was widely recognized long before identification of the Edg family LPA receptors that LPA stimulated cell proliferation in a PTX-sensitive manner, while it induced stress fiber formation and intracellu-

TABLE I. **Characteristics of Edg receptors.**

Receptor isoforms	New nomenclature	Expression	G protein coupling[a]	Signaling pathway
S1P receptors				
Edg1	$S1P_1$	widely distributed	Gi	MAPK[e]/AC inhibition/PLC[b]/Rac activation
Edg5	$S1P_2$	widely distributed	Gi, Gq, $G_{12/13}$	MAPK/cAMP increase[d]/PLC[c]/Rho/Rac inhibition
Edg3	$S1P_3$	widely distributed	Gi, Gq, $G_{12/13}$	MAPK/AC inhibition/PLC[c]/Rho/Rac activation
Edg6	$S1P_4$	lymphoid tissues, lung	Gi	MAPK/PLC[b]
Edg8	$S1P_5$	nervous tissue	Gi, $G_{12/13}$	AC inhibition
LPA receptors				
Edg2	LPA_1	widely distributed	Gi, Gq, $G_{12/13}$	MAPK/AC inhibition/PLC[c]/Rho
Edg4	LPA_2	nervous tissue, kidney, testis, lung, prostate	Gi, Gq, $G_{12/13}$	MAPK/AC inhibition/PLC[c]/Rho
Edg7	LPA_3	testis, prostate, heart, brain, lung, kidney	Gi, Gq	MAPK/AC inhibition/PLC[c]

[a]Determined by the sensitivity of the observed response to pertussis toxin (PTX) and by agonist-stimulated binding to G protein of GTP and its analogue. [b]Sensitive to PTX and observed in Chinese hamster ovary (CHO) cells. [c]Largely insensitive to PTX. [d]The precise mechanism is unknown. [e]MAPK, ERK mitogen–activated protein kinase; AC, adenylate cyclase; PLC, phospholipase C.

lar Ca^{2+} mobilization in a PTX-insensitive manner (*2, 3, 9, 30*).

The five Edg receptors specific for S1P are coupled to overlapping, yet distinct sets of intracellular signaling pathways. Edg1 is coupled exclusively *via* Gi/o to stimulation of MAPK and inhibition of adenylate cyclase. Edg1 is also coupled to stimulation of PLC largely *via* Gi/o in CHO cells. In this cell type, Edg1-mediated MAPK stimulation is totally dependent on Ras, but not protein kinase C (*20*). Edg3 and Edg5 are also coupled *via* Gi/o to Ras/MAPK like Edg1, but unlike Edg1, they are coupled to stimulation of PLC largely *via* PTX-insensitive Gq (*23, 24*). In addition, Edg3 and Edg5 but not Edg1 are coupled to Rho stimulation, as we determined by the direct measurement of a GTP-bound active form of RhoA with the pull-down assay using a Rho-binding domain of a Rho effector, rhotekin (*34*). What is more sophisticated with these S1P receptors is the fact that another small G protein Rac is bimodally regulated in isoform-specific manners (*28, 34*). Thus, we found that Edg1 and Edg3 mediate S1P stimulation of cellular Rac activity *via* Gi/o, whereas Edg5 mediates suppression of growth factor-stimulated Rac activity *via* a PTX-insensitive G protein, providing a molecular basis for S1P-mediated, bimodal regulation of cell migration (see below). S1P-induced Rac stimulation *via* Edg1 and Edg3 was dependent on PI 3-kinase. We recently demonstrated that Edg3-mediated PI 3-kinase stimulation was dependent on phospholipase D (*35*). Edg6 was reported to be coupled *via* Gi/o to stimulation of MAPK and phospholipase C and inhibition of adenylate cyclase, although its signaling mechanisms have not been as extensively studied as those of the other Edg receptors (*28, 30*). Edg8 was shown to be coupled to Gi and G_{12}, by determining ^{35}S-GTPγS binding to the G proteins. Edg8 mediated inhibition of adenylate cyclase in a PTX-sensitive manner, but unexpectedly did not stimulate MAPK.

III. Biological activities of the Edg receptors

LPA and S1P share many pleiotropic biological activities in a wide variety of cells. This appears to be due to both the similarities in the signal transduction mechanisms between the two receptor subfamilies as described above (Table I), and also frequent co-expression of both receptors in one cell type. However, it is important to note that certain activities are specific for S1P. These include promotion of formation of endothelial capillary–like structures and inhibition of tumor cell invasion and migration.

LPA is implicated in embryonic development of the nervous system. LPA stimulation of Edg2 in cultured cortical neuroblasts is reported to induce cell cycle–dependent morphological changes and cell migration (*5, 19, 30, 36*), suggesting roles for Edg2 in immature neuroblasts in the ventricular zone, where the expression of Edg2 is prominent. In postmitotic differentiating neurons, LPA may regulate migration of cells to their final destination and also modulate neurite formation, which may be mediated through Edg4 expressed in these differentiating neurons. In oligodendrocytes and Schwann cells, which are myelinating cells in the central and peripheral nervous tissues, stimulation of Edg2 activates the phosphatidylinositol (PI) 3-kinase/Akt cell survival signal pathway, leading to protection from apoptosis. Targeted deletion of the Edg2 gene in mice resulted in approximately 50% neonatal lethality, impaired suckling in neonatal pups, craniofacial dysmorphism, loss of LPA responsiveness in embryonic cerebral cortical neuroblasts in culture, and increased apoptosis in sciatic nerve Schwann cells (*36*). The impaired suckling may be brought about by altered development of the cerebral cortex or olfactory bulb and is probably responsible for increased neonatal death and reduced body size in survivors. Embryonic cortical neuroblasts also express another LPA receptor, Edg4, but this does not appear to rescue the abnormalities observed in Edg2 knockout mice, indicating a specific, non-redundant role of Edg2 in the developing cerebral cortex.

LPA is also suggested to play a role in cancer (*2, 3, 9*). LPA was shown to stimulate cell invasion in various tumor cell types. In addition, LPA stimulates cell proliferation in some tumor cells. Increased secretion of LPA may be a characteristic of some types of ovarian cancer, as suggested by the observation that LPA is present at a high concentration in ascites of ovarian cancer patients (*30*). Edg2/LPA_1 receptor is overexpressed in some malignant ovarian tumor cells, which may suggest an autocrine role for the LPA-Edg2 signaling system in ovarian cancer development.

Edg1 was initially cloned as an immediate early response gene in phorbol ester–stimulated vascular endothelial cells (*15*). S1P as well as phorbol ester promoted capillary-like network formation in the collagen gel endothelial cell cultures (*14*). Consistent with this activity of S1P, it acts on endothelial cells to stimulate cell migration and adherens junction assembly that involves upregulation of VE-cadherin (*14*). S1P stimulated angiogenesis *in vivo* in the matrigel plug assay. Vascular endothelial cells employed in these experiments expressed Edg1 and Edg3. The tube-forming action of S1P was attributed to both Edg1 and Edg3 by experiments employing antisense oligonucleotides. The tube-forming activity of S1P was also abolished by PTX and C3 toxin, indicating the critical contribution of Gi and Rho to the S1P-activated Edg receptor–mediated morphogenetic response of endothelial cells. Targeted deletion of the Edg1 gene did not compromise the initial step of blood vessel formation, *i.e.*, vasculogenesis (*37*). This might be due to redundant roles for Edg1 and Edg3, as suggested by the previous study showing the tube-forming action of S1P in vascular endothelial cells *via* both Edg1 and Edg3. In the Edg1 knockout mice, however, vascular maturation in later stages *in utero* was defective: smooth muscle cells failed to migrate to uniformly surround primitive capillaries, remaining at the ventral side of the vessels, which led to abnormal hemorrhage and intrauterine death. One explanation for this phenotype is that chemoattractant gradient is asymmetrically distributed and thereby smooth muscle migration is impaired. It remains unknown which cell type, particularly of endothelial cells and smooth muscle cells, depends on Edg1 function to activate smooth muscle migration.

One of the intriguing activities of S1P is its bimodal action on cell motility. It was first demonstrated that S1P inhibited chemotaxis of B16 melanoma cells very potently at a concentration as low as 10^{-8} M (*4, 38*). Consistent with inhibition of cell migration by S1P, it inhibited lamellipodia formation at the leading edge. It was also found that S1P inhibited motility of other types of tumor cells, as well as neutrophils and vascular smooth muscle cells (*4*). In sharp contrast, S1P directed cell migration of vascular endothe-

lial cells and embryonic fibroblasts (*14, 37*). The underlying mechanism for the bimodal actions of S1P on cell motility remained unresolved, although a pioneering work by Igarashi *et al.* (*4, 38*) strongly suggested cell surface rather than intracellular action of S1P for its inhibitory regulation. We demonstrated in a heterologous expression system using CHO cells that among the widely expressed types of S1P receptors, Edg1 and Edg3 mediated robust chemotaxis toward S1P, whereas Edg5 mediated marked inhibition of chemotaxis toward other attractants such as insulin-like growth factor–I (IGF I) (*28, 34*). S1P stimulation of Edg5 by itself did not stimulate or inhibit cell migration. Importantly, negative regulation of cell migration mediated by Edg5 in IGF I–stimulated cells was dependent on the concentration gradient of S1P. We demonstrated for the first time that repulsive Edg5 mediated inhibition of Rac activity *via* a PTX-insensitive G protein, in sharp contrast to the attractive receptors Edg1 and Edg3, which mediate S1P stimulation of Rac activity *via* a PTX-sensitive Gi/o. We also found that Edg3 and Edg5, but not Edg1, mediated stimulation of RhoA. The activity of Cdc42, the third member of the Rho family small G proteins, was not affected by either of the three Edg receptors. The expression of dominant negative mutants of each of the Rho family GTPases in CHO cells revealed that cell migration was dependent on Rac and Cdc42. Thus, these observations together indicate that the attractant receptors Edg1 and Edg3, and the repulsive receptor Edg5 respectively regulate the activity of Rac positively and negatively, and thereby regulate cell migration in opposite directions. Consistent with this notion, we observed that S1P inhibited Rac activity and cell motility *via* Edg5 (Arikawa, K. *et al.*, unpublished observation) in adult rat and human aortic vascular smooth muscle cells (*39*) and B16 melanoma cells. These cells abundantly express Edg5 but not Edg1. In contrast, in human umbilical vein endothelial cells, in which Edg5 is essentially absent, Edg1 and, to a lesser extent, Edg3 mediate S1P stimulation of migration. Overexpression of Edg5 in adult aortic smooth muscle cells converted their S1P responsiveness from negative to positive mode in terms of both migration and regulation of cellular Rac activity (*39*). Thus, Edg5 is the first receptor to be identified that negatively regulates cellular Rac activity. We also found that its mechanism of action for inhibition of Rac involves stimulation of a GTPase-activating protein (GAP), but not inhibition of a guanine nucleotide exchange factor (GEF) (*37*).

IV. Future prospects

In view of their growth factor–like activities and regulatory activities on cell motility and cytoskeletal structures (*2, 3, 9, 27, 28, 30, 34, 38*), the lysophospholipid mediators probably play roles in both physiological and pathological processes, the latter including inflammation, tumor invasion and metastasis, and vascular remodeling. Both LPA and S1P probably act as blood-born circulating mediators as well as locally produced autocrine and/or paracrine mediators. Regulation of synthesis of the lysophospholipids under physiological and pathological conditions will be a subject of intense investigation. Development of specific antagonists and agonists for Edg receptor isoforms and inhibitors of synthesis and degradation of the lysophospholipids should not only help to reveal the physiological and pathological roles of the lysophospholipids, but also lead to development of novel therapeutic strategies for various diseases.

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