Nrg-1 Belongs to the Endothelial Differentiation Gene Family of G Protein-coupled Sphingosine-1-phosphate Receptors*

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The previously cloned rat nerve growth factor-regulated G protein-coupled receptor NRG-1 (Glickman, M., Malek, R. L., Kwitek-Black, A. E., Jacob, H. J., and Lee N. H. (1999) Mol. Cell. Neurosci. 14, 141–52), also known as EDG-8, binds sphingosine-1-phosphate (SIP) with high affinity and specificity. In this paper we examined the signal transduction pathways regulated by the binding of SIP to EDG-8. In Chinese hamster ovary cells heterologously expressing EDG-8, SIP inhibited forskolin-induced cAMP accumulation and activated c-Jun NH2-terminal kinase. Surprisingly, SIP inhibited serum-induced activation of extracellular regulated protein kinase 1 and 2 (ERK1/2). Treatment with pertussis toxin, which ADP-ribosylates and inactivates Gi, blocked SIP-mediated inhibition of cAMP accumulation, but had no effect on c-Jun NH2-terminal kinase activation or inhibition of ERK1/2. The inhibitory effect of SIP on ERK1/2 activity was abolished by treatment with orthovanadate, suggesting the involvement of a tyrosine phosphatase. A subunit selective [35S] guanosine 5’-O-(thio)triphosphate binding assay demonstrates that EDG-8 activated G12 and G13 but not G0 and Gq/11 in response to SIP. In agreement, EDG-8 did not stimulate phosphoinositide turnover or cAMP accumulation. The ability of SIP to induce mitogenesis in cells expressing the EDG-1 subfamily of G protein-coupled receptors is well characterized. In contrast, SIP inhibited proliferation in Chinese hamster ovary cells expressing EDG-8 but not empty vector. The antiproliferative effect, like SIP-mediated ERK1/2 inhibition, was orthovanadate-sensitive and pertussis toxin-insensitive. Our results indicate that EDG-8, a member of the EDG-1 subfamily, couples to unique signaling pathways.

The lyosphospholipids sphingosine-1-phosphate (SIP)1 and lysophosphatidic acid (LPA) are endogenous ligands of the EDG family of G protein-coupled receptors (GPCRs) (1–3). Presently, eight EDG receptors have been cloned and are divided into two or three homology clusters or subfamilies (4–13). EDG-1, -3, -5, and -8 exhibit high sequence homology to one another and have high affinity for SIP (14–17). EDG-2, -4, and -7 form a second cluster and are high affinity receptors for LPA (1, 5, 8, 18). EDG-6 displays intermediate homology between the two clusters and binds SIP with moderate to high affinity (7, 19).

SIP-activated EDG receptors are coupled to multiple effector pathways, including activation/inhibition of adenyl cyclase, stimulation of phosphoinositide (PI) hydrolysis, mobilization of Ca2+ (20–26), induction of DNA synthesis (27–29), and stimulation of the MAP kinase family members ERK1/2, SAPK/JNK, and p38 (17, 20–24). For example, activation of EDG-1, -3, or -5 by SIP leads to a pertussis toxin (PTX)-sensitive stimulation of ERK1/2 (17, 20–22, 24), suggesting the involvement of G0 or G12 proteins. In contrast, EDG-5 stimulates both stress-activated protein kinases, SAPK/JNK and p38, in a PTX-insensitive manner (23), indicating that EDG receptor coupling to MAP kinase family members involves PTX-sensitive and -insensitive G proteins. In addition to the differential coupling of G proteins and EDG receptors to MAP kinases, there exists differential coupling to the adenyl cyclase-cAMP and phospholipase C-Ca++ systems (20–26). EDG-1 couples exclusively to G0, whereas EDG-3 and EDG-5, in addition to G0, couple to G12 and Gq/11 (25, 30).

Although the biological functions of the EDG-1 subfamily of receptors are not well understood, binding of SIP to EDG-1 and -5 has been recently shown to regulate migration (15, 17, 25, 29) and angiogenesis (29, 31). More recently, EDG-1, -3, and -5 have been linked to induction of cell proliferation (32). Interestingly, EDG-8 is closely related to EDG-5, exhibiting 40–44% sequence identity. Edg-8, which was initially cloned from rat pheochromocytoma cells as the nerve growth factor-regulated orphan GPCR nrg-1 (6, 33), has been shown to be in close proximity to edg-5 on rat chromosome 8 (6), possibly because of a gene duplication event. Thus, it is of great interest to examine the signaling pathways regulated by SIP binding to EDG-8 and its potential biological consequences. Unexpectedly we found ovary; EDG, endothelial differentiation gene; MAP, mitogen-activated protein; ERK, extracellular regulated protein kinase; PI, phosphoinositide; SAPK/JNK, stress-activated protein kinase/c-Jun NH2-terminal kinase; PAG, polyacrylamide gel electrophoresis; GTP-S, guanosine 5’-3’-O-(thio)triphosphate; HA, hemagglutinin; AT1, angiotensin II type 1; AT2, angiotensin II type 2.

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1 The abbreviations used are: SIP, sphingosine-1-phosphate; LPA, lysophosphatidic acid; SPC, sphingosylinphosphocholine; GPCR, G protein coupled receptor; PTX, pertussis toxin; CHO, Chinese hamster ovary; EDG, endothelial differentiation gene; MAP, mitogen-activated protein; ERK, extracellular regulated protein kinase; PI, phosphoinositide; SAPK/JNK, stress-activated protein kinase/c-Jun NH2-terminal kinase; PAGE, polyacrylamide gel electrophoresis; GTP-S, guanosine 5’-3’-O-(thio)triphosphate; HA, hemagglutinin; AT1, angiotensin II type 1; AT2, angiotensin II type 2.

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that EDG-8 is mainly coupled to G\(_i\) and G\(_q\) and inhibits, rather than stimulates, ERK activation in CHO cells. Moreover, SIP markedly inhibits proliferation in CHO cells overexpressing EDG-8 in a G\(_i\)-independent manner.

**EXPERIMENTAL PROCEDURES**

**Materials**—CHO cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Ham’s F-12 medium containing 10% charcoal-treated dialyzed fetal bovine serum (Life Technologies, Inc.) as described previously (33–35). Dialyzed serum treated with activated charcoal removes all contaminating lipids such as SIP (33–35) and was used in all experiments involving serum starvation. Pertussis toxin, isobutylmethylxanthine, and forskolin were from Sigma. SIP and Go4, polyclonal antibody was from Calbiochem (San Diego, CA). Lipids other than SIP, which was from Biomol (Plymouth Meeting, PA), were from Avanti Polar Lipids (Birmingham, AL). Phospho-JNK MAP kinase (G-7), c-Myc (9E10), and HA probe (Y-11) antibodies and GST-ATF-2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-ERK1/2, phospho-p38, ERK1/2, SAPK/JNK, and p38 MAP kinase antibodies and Elk-1 and c-Jun fusion proteins were from New England Biolabs (Beverly, MA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies were from Upstate Biotechnology (Lake Placid, NY).

**Transfection of Cells with HA-tagged EDG-8**—A polymerase chain reaction strategy was used to insert a 9-amino acid HA epitope sequence (YPYDVPDYASL) into the COOH terminus of EDG-8. The sense and antisense polymerase chain reaction primers were 5’-aaa-aagcttAAAGGTCGACTGAGCAG-3’ and 5’-aaaactgcgttCAAATGCCAGAATGC-3’, respectively. The sense primer has 21 nucleotides of 5’ untranslated region from the rat EDG-8 cDNA (6). The antisense primer has 27 nucleotides coding for the HA epitope and 7 codons from the COOH terminus of EDG-8 (bold type). Polymerase chain reaction products were cloned into the HindIII and XhoI cloning sites of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA), generating HAedg8pcDNA3. The fidelity of the plasmid construct was verified by sequencing.

**Stable Transfection**—CHO cells were stably transfected with HAedg8pcDNA3 using LipofectAMINE 2000 (Life Technologies, Inc.). Expression of EDG-8 in transfected cells was verified by immunoblotting using the HA polyclonal antibody.

**[32P]SIP Binding**—[32P]SIP was prepared as described previously (36). CHO cells seeded at a density of 75,000 cells/cm\(^2\) were transiently transfected with HAedg8pcDNA (1 μg/assay point) and the indicated concentrations of [32P]SIP. Unlabeled lipid competitors were added as 4 mg/ml fatty acid-free bovine serum albumin albumus complexes, and bound [32P]SIP was quantitated by scintillation counting as described previously (36).

**Measurement of cAMP Production**—CHO cells were grown in 12-well plates, transiently transfected with HAedg8pcDNA3 or the human β2-adrenergic receptor cDNA in pSVL (Stratagene, La Jolla, CA) and grown in Ham’s F-12 medium supplemented with 10% charcoal-treated dialyzed fetal bovine serum. After 3 days, CHO cells were either transiently (2 days) or stably transfected with HAedg8pcDNA3 using LipofectAMINE 2000 (life Technologies, Inc.). Expression of EDG-8 in transfected cells was verified by immunoblotting using the HA polyclonal antibody.

**Measurement of cAMP Accumulation**—CHO cells were grown in 35-mm tissue culture dishes, treated with the indicated lipids, harvested/lysed in 95 °C SDS-PAGE sample buffer as described previously (34). Proteins were separated on 10% polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Blots were probed with phosphorylation state-specific MAP kinase primary antibodies. Goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies allowed detection of proteins by the ECL Plus Detection System (Amersham Pharmacia Biotech). Fluorograms were quantitated by densitometry. Blots were stripped, reprobed with primary antibodies that recognize MAP kinases independently of phosphorylation state, and quantitated. Data are expressed as the means ± S.E. of n independent experiments.

**Immunocomplex Kinase Assays**—Transiently transfected CHO cells were treated with the indicated lipids, washed in ice-cold phosphate-buffered saline, lysed in IP Buffer (20 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 1 mM EDTA, 0.5% (v/v) Nonidet P-40, 1% Triton X-100, 2 mM sodium orthovanadate, 20 μg/ml aprotinin, 5 μg/ml leupeptin, 50 μg/ml sodium fluoride), and disrupted by aspiration through a 21-gauge needle. ERK1/2-enzyme conjugation reactions were incubated with MAP kinase antibody for 2 h at 4 °C. Immunocomplexes were precipitated with IP Buffer equilibrated protein A-agarose (Sigma) for 2 h at 4 °C, washed three times with IP Buffer, washed twice with kinase buffer (25 mM HEPES, pH 7.5, 1 mM MgCl\(_2\), 25 mM sodium β-glycerophosphate, 2 mM sodium orthovanadate, 0.5 mM dithiothreitol), and resuspended in kinase buffer containing 100 μM ATP, 5 μCi of [γ-32P]ATP. For measurement of ERK1/2, SAPK/JNK, or p38 MAP kinase activities, immunocomplexes were incubated with 2 μg of Elk-1 fusion protein, c-Jun fusion protein, or GST-ATF-2 substrate, respectively. Reactions were incubated 30 min at 30 °C and terminated by the addition of 2× SDS-PAGE loading buffer. Proteins were separated on a 10% SDS-PAGE gel and analyzed by autoradiography.

**RESULTS**

**[32P]SIP Binds to EDG-8**—Many cell lines commonly employed for heterologous expression of receptor genes, including COS and HEK293 cells, respond to S1P with Gi/o-independent effects of S1P on proliferation. S1P and inhibitors were added at the indicated concentrations of [32P]SIP on 10% charcoal-treated dialyzed fetal bovine serum. After 18 h (t = 0), cells were washed twice with Ham’s F-12 and grown in Ham’s F-12 without serum to measure proliferation. CHO cells were transiently (2 days) or stably transfected with HAedg8pcDNA3 or vector alone and plated in 12-well plates containing Ham’s F-12 medium supplemented with 10% charcoal-treated dialyzed fetal bovine serum. After 18 h (t = 0), cells were washed twice with Ham’s F-12 and grown in Ham’s F-12 without serum to measure proliferation. CHO cells were transiently (2 days) or stably transfected with HAedg8pcDNA3 or vector alone and plated in 12-well plates containing Ham’s F-12 medium supplemented with 10% charcoal-treated dialyzed fetal bovine serum. After 18 h (t = 0), cells were washed twice with Ham’s F-12 and grown in Ham’s F-12 without serum to measure proliferation. S1P and inhibitors were added at the indicated concentrations. After 48 h, cells were washed with phosphate-buffered saline, fixed with 70% ethanol for 10 min, and stained with crystal violet. Incorporated dye was dissolved in 0.1 M sodium citrate in 0.1 M sodium hydroxide, and the absorbance was measured at 540 nm in a hemocytometer. Cell numbers in four random microscopic fields were counted from each well. Measurements from the two methods gave identical results. Each determination represents the mean ± S.E. of three to four individual wells.

**SBS-PAGE and Immunoblotting**—Transiently transfected CHO cells were grown in 35-mm tissue culture dishes, treated with the indicated lipids, harvested/lysed in 95 °C SDS-PAGE sample buffer as described previously (34). Proteins were separated on 10% polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Blots were probed with phosphorylation state-specific MAP kinase primary antibodies. Goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies allowed detection of proteins by the ECL Plus Detection System (Amersham Pharmacia Biotech). Fluorograms were quantitated by densitometry. Blots were stripped, reprobed with primary antibodies that recognize MAP kinases independently of phosphorylation state, and quantitated. Data are expressed as the means ± S.E. of n independent experiments.
I and II). Following transient transfection with an expression vector for HA-tagged EDG-8, CHO cells specifically bound \(^{32}P\)S1P with high affinity (\(K_d = 6 \pm 4\) nM; \(n = 3\) independent experiments; Fig. 1, left panel). This \(K_d\) value is in excellent agreement with a recent study performed in HEK293T cells (14), demonstrating that EDG-8 is indeed a high affinity receptor for S1P in diverse cell types. The level of expression of EDG-8 in CHO cells based on \(^{32}P\)S1P binding was comparable with that seen in these cells transfected with EDG-1, -3, and -5 (25). In agreement with a recent report in HEK293T cells (14), only unlabeled S1P and dihydro-S1P effectively competed with \(^{32}P\)S1P for binding to EDG-8 (Fig. 1, right panel), whereas LPA, sphingosine, and SPC had no significant effects.

**Effect of EDG-8 on cAMP Formation**—Previously it has been shown that S1P treatment of EDG-8-transfected CHO cells increases cAMP content in a PTX-insensitive manner with the following rank order of efficacy: EDG-5 > EDG-3 > EDG-1 (25). Treatment of CHO cells transiently expressing EDG-8 with S1P (1 to 1000 nM), however, had no effect on intracellular cAMP content (Table I), whereas isoproterenol (2 \(\mu\)M) caused a robust 6-fold increase in cAMP levels in CHO cells expressing the \(\beta_2\)-adrenergic receptor. To assess the ability of EDG-8 to mediate inhibition of cAMP accumulation, cAMP levels were increased by treatment with forskolin. Addition of S1P resulted in a dose-dependent, PTX-sensitive, decrease in forskolin-induced cAMP accumulation (Fig. 2A), whereas in empty vector-transfected cells, S1P, even at a concentration as high as 1 \(\mu\)M, had no significant effect on forskolin-induced cAMP accumulation (Fig. 2B). Similar to S1P, dihydro-S1P (1 \(\mu\)M) effectively inhibited cAMP formation, whereas low concentrations of LPA, sphingosine, and SPC (10 to 100 nM) had no significant effect, and only small (22%) inhibition was found at a high concentration of LPA (1 \(\mu\)M) (Fig. 2B).

**Effect of EDG-8 on PI Turnover**—In CHO cells transfected with EDG-1, -3, or -5, S1P increases intracellular Ca\(^{2+}\) concentrations caused by the activation of phospholipase C (25). In contrast, S1P, at concentrations ranging from 1 nM to 1 \(\mu\)M, did not significantly stimulate PI turnover in EDG-8-expressing cells (Table I), whereas carbachol (1 mM) produced a marked 5 \(\pm\) 0.9-fold increase in PI turnover in cells transiently expressing the \(M_1\)-muscarinic receptor known to stimulate phospholipase C via \(G_{\alpha1}\) (\(n = 3\) independent experiments).

**EDG-8 Mediates Repression of Serum-activated ERK1/2 in a PTX-insensitive but Phosphatase-dependent Manner**—Because several EDG receptors have been reported to activate ERK1/2 (17, 20, 21, 23, 42), we investigated whether S1P-mediated stimulation of EDG-8 led to a similar response. The serine/threonine kinases ERK1/2, SAPK/JNK, and p38 belong to the MAP kinase superfamily, share sequence homology, and are activated upon phosphorylation of homologous threonine and tyrosine residues by dual specificity MAP kinase kinases. Thus, the phosphorylation state of a MAP kinase family member reflects its activation state (43–47).

In serum-starved EDG-8-expressing CHO cells, S1P did not activate ERK1/2 as measured by either Western blot analysis with phosphorylation state-specific antibodies or immunocomplex kinase assay of whole cell lysates (Table I). Even after prolonged incubations of up to 60 min, S1P had no significant effect on ERK activation. Surprisingly, induction of ERK1/2 phosphorylation by serum was repressed in a time-dependent fashion by S1P (1 \(\mu\)M) in CHO cells expressing EDG-8 (Fig. 3A). This inhibitory response was insensitive to PTX (Fig. 3A). Furthermore, serum-induced ERK1/2 phosphorylation was not inhibited by S1P in CHO cells transfected with empty vector (Fig. 3A). Similar to S1P, dihydro-S1P inhibited ERK1/2 phosphorylation by \(\approx 40\%\) (Fig. 3B). In agreement with their inability to bind to EDG-8, LPA, SPC, and sphingosine had no effects on ERK phosphorylation induced by serum (Fig. 3B). Similar results were obtained by immunocomplex kinase assays (Fig. 4). Once more, S1P but not LPA repressed serum activation of ERK1/2 by \(\approx 50–60\%\).

In contrast, in CHO cells transiently expressing EDG-1, -3, or -5, S1P did not inhibit serum-induced phosphorylation of ERK1/2 as determined by Western blot analysis, even in cells pretreated with PTX (Table I). Identical results were noted by immunocomplex kinase assays (Table I).

Although the binding of S1P to EDG-1, -3, and -5 did not inhibit ERK activation, this is not unprecedented. It has previously been demonstrated that the GPCR, angiotensin II type 2 (AT\(_2\)) receptor, inhibits serum-activated ERK1/2 in several cell types (48, 49). This effect was abolished by inhibition of protein phosphatases. Similarly, the ability of S1P to inhibit serum-activated ERK 1/2 in EDG-8-expressing CHO cells was blocked by orthovanadate and okadaic acid, albeit to a lesser extent (Fig. 4). Neither okadaic acid (5 \(\mu\)M) nor orthovanadate (0.1 mM) alone had a significant effect on serum-stimulated ERK1/2 activation (Fig. 4).

**EDG-8 Activates SAPK/JNK but Not p38 MAP Kinase**—In serum-starved CHO cells transiently expressing EDG-8, S1P induced a time- (Fig. 5A) and dose-dependent (EC\(_{50}\) = 100 nM; data not shown) activation of JNK as measured by an increase in JNK phosphorylation. Increased JNK phosphorylation was apparent as early as 1 min after treatment with S1P (\(3 \pm 0.2\)-fold over basal; \(n = 3\) independent experiments) and was sustained for at least 60 min. Remarkably, only the p54 isoform of JNK appears to be phosphorylated in a PTX-independent manner, suggesting that EDG-8 may regulate specific JNK isoforms. S1P-stimulated JNK phosphorylation was not observed in parental CHO cells transfected with empty vector (Fig. 5A). This phosphorylation was associated with a 2.8-fold increase in JNK activity (Fig. 6). Similar to S1P, dihydro-S1P also stimulated p54 JNK phosphorylation 2.4-fold, whereas other lysophospholipids did not have any significant effects (Fig. 5B). In agreement with the Western analyses (Fig. 5B), in vitro kinase assays demonstrated that LPA does not stimulate...
In EDG-5 transfected CHO cells, S1P did not promote \([^{35}S]GTP\) binding and activation of heterotrimeric G proteins as determined by immunocomplex kinase assay. In contrast to its effect in EDG-8-expressing cells, S1P resulted in a 2.3- to 2.5-fold increase in G\(a_{12}\) activation in EDG-8 transfected CHO cells treated with S1P. In EDG-8-expressing cells, however, S1P treatment produced a 2- to 2.5-fold increase in \([^{35}S]GTP\) binding and activation of G\(a_{12}\) proteins (Fig. 7). Thus, the lack of detectable coupling to G\(a_{12}\) in CHO cells expressing EDG-8 is not due to the absence of these G proteins in CHO cells. Moreover, when membranes from CHO cells expressing the \(\beta_2\)-adrenergic receptor were incubated with 2 \(\mu M\) isoproterenol, a 2.9 \(\pm\) 0.4-fold increase in \([^{35}S]GTP\) binding to G\(a_{12}\) was observed (n = 2 independent experiments).

**Fig. 2. Inhibition of cAMP accumulation by EDG-8.** CHO cells transiently transfected with EDG-8 were pretreated with saline vehicle or PTX (100 ng/ml, 18 h). Cells were pretreated with 0.5 \(\mu M\) isobutylmethylxanthine for 30 min and incubated with the indicated concentrations of S1P (A) or related lipids (B) in the presence of 10 \(\mu M\) forskolin. Results are the means \(\pm\) S.E. of three to six independent experiments. An asterisk indicates a statistically significant difference compared with forskolin treatment in the absence of S1P as determined by Student's t test (p < 0.05). SPC, sphingosylphosphorylcholine; \(dh\)-SIP, dihydro-SIP; Sph, sphingosine.

JNK activity in EDG-8-expressing CHO cells (Fig. 6). In contrast to its effect on JNK activity in EDG-8 overexpressing CHO cells, S1P has no significant effect on p38 phosphorylation (data not shown; 1 \(\mu M\) S1P; 1–60 min). Immunocomplex kinase assays also confirmed the inability of S1P/EDG-8 to induce p38 activity, whereas the positive control arsenite (50 \(\mu M\); 60 min) potently activated p38 in CHO cells (data not shown).

**Table I**

| EDG receptor effector pathways | AC/cAMP | PLC/Ca\(^2^+\) | ERK | JNK | p38 | Proliferation |
|--------------------------------|---------|----------------|------|-----|-----|--------------|
| EDG-1                          | \(\uparrow\)\(\uparrow\)
| EDG-3                          | \(\uparrow\)\(\uparrow\) | ND   | ND  | ND  | Refs. 17, 20, 21, 25, 26, 28, and 36 |
| EDG-5                          | \(\uparrow\)\(\uparrow\) | ND   | ND  | ND  | Refs. 20, 25, 26, 42, and 63 |
| EDG-8                          | \(\uparrow\)\(\uparrow\) | \(\uparrow\)\(\uparrow\) | \(\uparrow\)\(\uparrow\) | \(\uparrow\)\(\uparrow\) | Present study |

* \(\uparrow\) positive effect; \(\downarrow\) negative effect; rank order of intrinsic activity is \(\uparrow\) \(\uparrow\) > \(\uparrow\) \(\uparrow\) > \(\uparrow\) \(\uparrow\), positive and negative effects reported. —, no effect; ND, not determined.

1. Activation of adenyl cyclase was measured by determining cAMP levels in the presence of IBMX after treatment with 1 \(\mu M\) S1P for 20 min.
2. PI turnover was measured as described under "Experimental Procedures."

**Table II**

| Differential coupling of EDG receptors to heterotrimeric G proteins |
|---------------|----------------|----------------|-------------|----------------|----------------|----------------|
|               | Go\(_i\) | Go\(_o\) | Go\(_ni\) | Go\(_o12\) | Go\(_o13\) | References |
| EDG-1          | —(*) | —(*) | —(*) | —(*) | —(*) | Refs. 30° and 22° |
| EDG-3          | —(*) | +(*) | +(*) | +(*) | ND | Ref. 30° |
| EDG-5          | —(*) | +(*) | +(*) | ND | +(*) | Ref. 30° |
| EDG-8          | —(*) | +(*) | +(*) | ND | ND | Present study |

* indicates no coupling; + indicates positive coupling; ND, not determined.

1. S1P promotes association of the indicated G proteins based on \([^{35}S]GTP\) binding in S9 or HEK293 cell membranes.

2. EDG-1 co-immunoprecipitates Go\(_o12\), Go\(_o\), and Go\(_n11\) polypeptides.

3. S1P (1 \(\mu M\)) promotes \([^{35}S]GTP\) binding to Go\(_o\) family and Go\(_o11\) but not Go\(_o\), or Go\(_n11\) through EDG-8 in CHO cell membranes. CHO cells express only trace levels of Go\(_o13\), for which no activation could be discerned through EDG-8. EDG-5, a known activator of Go\(_o13\) (30), was used as a positive control. In EDG-5 transfected CHO cells, S1P did not promote \([^{35}S]GTP\) binding to Go\(_o13\).
that G_i/o proteins may be involved in the antiproliferative response induced by S1P. To this end, cells were pretreated with PTX. PTX was completely ineffective in abolishing S1P-induced anti-proliferation (Fig. 8). In contrast, treatment of EDG-8-transfected cells with orthovanadate (1 mM) significantly impaired S1P-induced antiproliferative effects, suggesting that this response is dependent on a protein-tyrosine phosphatase (Fig. 8). Neither PTX nor orthovanadate had deleterious effects on CHO overexpressing EDG-8 cells as demonstrated by normal attachment and survival of the cells after 48 h of treatment (data not shown).

**DISCUSSION**

This study demonstrates that EDG-8, originally cloned as a GPCR termed NRG-1 (6), binds and is activated by S1P when heterologously expressed in CHO cells. CHO cells were selected to characterize EDG-8 because this cell line does not readily respond to S1P in the absence of exogenous EDG gene transfer (20, 23, 25). Furthermore, many studies relating to EDG-1, -3, and -5 signaling have been performed in CHO cells, thereby providing a common background for direct comparison to EDG-8 signaling (Tables I and II). The affinity of S1P for EDG-8 (K_d = 6 nM) is similar to previously reported affinity values obtained for EDG-1, -3, and -5 (K_d = 8–26 nM) (15, 25). Based upon the lysosphospholipid ligands that were tested for their ability to displace [32P]S1P binding, the pharmacological profile of EDG-8 closely resembles other S1P-binding EDG-1 receptors. Moreover, similar to our previous results with EDG-1, -3, and -5, SPC (15, 36) did not compete with [32P]S1P binding via EDG-3, EDG-5, and possibly EDG-1 (27, 29, 42). Thus, it was of great interest to examine whether binding of S1P to EDG-8 also regulates mitogenic pathways. To assess induction of proliferation, stably transfected cells plated in 12-well plates containing serum-free medium were counted after treatment with 1 mM S1P for 48 h. Cell numbers from both control vector and EDG-8 transfectants did not increase in response to S1P (data not shown), suggesting that S1P does not behave as a mitogen at EDG-8.

Interestingly, the proliferative response of EDG-8-transfected CHO cells was 62% lower than vector-transfected cells following mitogenic stimulation with 10% serum for 48 h (Fig. 8). This observation was corroborated by independent experiments, demonstrating that EDG-8-transfected CHO cells exhibit a 3-fold reduction in [3H]thymidine incorporation compared with untransfected or vector-transfected cells (data not shown). Taken together, our findings suggest that EDG-8 contains intrinsic activity and inhibits serum-stimulated cell proliferation. Furthermore, addition of 1 mM S1P significantly inhibited serum-stimulated proliferation of EDG-8-transfected cells (Fig. 8). The anti-proliferative effect induced by S1P binding to EDG-8 was dose-dependent with an EC50 value around 20 nM (data not shown). In contrast, 1 mM S1P treatment did not inhibit proliferation of vector-transfected cells stimulated with 10% serum (Fig. 8). Because biochemical evidence indicates that EDG-8 couples to G_i/o, we investigated the possibility that Gi/o proteins may be involved in the antiproliferative response induced by S1P. To this end, cells were pretreated with PTX. PTX was completely ineffective in abolishing S1P-induced anti-proliferation (Fig. 8). In contrast, treatment of EDG-8-transfected cells with orthovanadate (1 mM) significantly impaired S1P-induced antiproliferative effects, suggesting that this response is dependent on a protein-tyrosine phosphatase (Fig. 8). Neither PTX nor orthovanadate had deleterious effects on CHO overexpressing EDG-8 cells as demonstrated by normal attachment and survival of the cells after 48 h of treatment (data not shown).

**DISCUSSION**

This study demonstrates that EDG-8, originally cloned as a GPCR termed NRG-1 (6), binds and is activated by S1P when heterologously expressed in CHO cells. CHO cells were selected to characterize EDG-8 because this cell line does not readily respond to S1P in the absence of exogenous EDG gene transfer (20, 23, 25). Furthermore, many studies relating to EDG-1, -3, and -5 signaling have been performed in CHO cells, thereby providing a common background for direct comparison to EDG-8 signaling (Tables I and II). The affinity of S1P for EDG-8 (K_d = 6 nM) is similar to previously reported affinity values obtained for EDG-1, -3, and -5 (K_d = 8–26 nM) (15, 25). Based upon the lysosphospholipid ligands that were tested for their ability to displace [32P]S1P binding, the pharmacological profile of EDG-8 closely resembles other S1P-binding EDG-1 receptors. Moreover, similar to our previous results with EDG-1, -3, and -5, SPC (15, 36) did not compete with [32P]S1P binding via EDG-3, EDG-5, and possibly EDG-1 (27, 29, 42). Thus, it was of great interest to examine whether binding of S1P to EDG-8 also regulates mitogenic pathways. To assess induction of proliferation, stably transfected cells plated in 12-well plates containing serum-free medium were counted after treatment with 1 mM S1P for 48 h. Cell numbers from both control vector and EDG-8 transfectants did not increase in response to S1P (data not shown), suggesting that S1P does not behave as a mitogen at EDG-8.
for binding to EDG-8. This is not surprising because recently a unique receptor for SPC, known as ORG1, has been identified (50). In contrast, others have previously demonstrated that SPC activates endogenous Gi in HEK293 cells heterologously expressing EDG-1 (30) and induces Ca\(^{2+}\) mobilization in CHO cells transfected with EDG-5 (23). Taken together, the binding data presented here establish that EDG-8 is a specific S1P and
EDG-8 resembles EDG-1 by inhibiting adenylyl cyclase via a PTX-sensitive mechanism in CHO cells, implicating G\(_i/G\_o\) proteins in the signaling process. \(^{[35S]}\)GTP\(_s\) binding assays corroborate the coupling of EDG-8 with G\(_i\). Interestingly, LPA at a relatively high concentration (1 \(\mu\)M) was able to weakly inhibit \(\alpha\)MIP formation in CHO cells expressing EDG-8. The ability of LPA to weakly inhibit \(\alpha\)MIP formation but not displace \(^{[32P]}\)S1P binding in EDG-8-transfected cells (present study) is reminiscent of findings that LPA does not displace \(^{[32P]}\)S1P binding in EDG-1-transfected cells but promotes ERK1/2 (51). It has been proposed that EDG-1 contains distinct sites for S1P and LPA (51). Our data with EDG-8 supports a similar two-site model. However, this hypothesis awaits definitive characterization of the binding sites by site-directed mutagenesis. Notwithstanding, our displacement binding studies suggest that EDG-8 is not an LPA receptor. Identical results were obtained in HEK293T cells overexpressing EDG-8 (14). Indeed, it has been convincingly demonstrated that LPA is the high affinity ligand for EDG-2, -4, and -7 (1, 5, 8).

Although CHO cells transfected with EDG-1, -3, or -5 can activate ERK in response to S1P in a PTX-sensitive and Ras-dependent manner (17, 20, 23, 24), we found instead that S1P inhibited ERK activity in EDG-8-transfected CHO cells. Although the majority of GPCRs studied to date (including the EDG receptors) appear to couple positively with ERK1/2 (52), examples of GPCR-mediated repression of ERK1/2 activity have been reported (48, 49). In cultured neurons, AT\(_1\) receptors activate ERK1/2, whereas AT\(_2\) receptors inhibit serum-activated ERK1/2 (48). Considering that the MAP kinases are important in growth, differentiation, and apoptosis (52), it has been proposed that the antagonistic modulation of ERK activity by different receptors within the same gene family acts as a molecular counterbalance system (48). Hence, the ability of EDG-1, -3, and -5 to stimulate ERK1/2 activity and EDG-8 to repress it parallels the AT\(_1/AT\_2\) receptor system. The finding that expression of EDG-8 but not EDG-1 or EDG-3 in PC12 cells is chronically down-regulated by growth factors (6) provides a potential mechanism for fine tuning this counterbalance system. Such a scenario has been demonstrated in the AT receptor family, where AT\(_2\) receptor expression is up-regulated following cellular injury to antagonize AT\(_1\) signaling (53).

The exact physiological role of ERK inhibition by GPCRs remains to be elucidated. EDG-1, -3, and -5 have been demonstrated to mediate PTX-sensitive cell proliferation (28, 42) in PC12 cells is another distinguishing feature of the signaling pathways. While this manuscript was in review, Im et al. (14) described the recloning of NRG-1 (EDG-8) from rat brain. In that report, EDG-8 was shown to bind S1P, inhibit forskolin-stimulated cAMP accumulation in rat hepatoma Rh7777 cells, and couple to G\(_i\) in Xenopus laevis oocytes, in agreement with our data. In conclusion, we have demonstrated fundamental differences in G protein coupling between the four known high affinity S1P receptors, EDG-1, -3, -5, and -8. Furthermore, we have identified differences in effector pathways that are potentially related to differences in G protein coupling.
Nrg-1 Belongs to the Endothelial Differentiation Gene Family of G Protein-coupled Sphingosine-1-phosphate Receptors
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