Sphingosine-1–Phosphate as a Ligand for the G Protein–Coupled Receptor EDG-1

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The sphingolipid metabolite sphingosine-1–phosphate (SPP) has been implicated as a second messenger in cell proliferation and survival. However, many of its biological effects are due to binding to unidentified receptors on the cell surface. SPP activated the heterotrimeric guanine nucleotide binding protein (G protein)–coupled orphan receptor EDG-1, originally cloned as *Endothelial Differentiation Gene–1*. EDG-1 bound SPP with high affinity (dissociation constant = 8.1 nM) and high specificity. Overexpression of EDG-1 induced exaggerated cell-cell aggregation, enhanced expression of cadherins, and formation of well-developed adherens junctions in a manner dependent on SPP and the small guanine nucleotide binding protein Rho.

Morphogenetic differentiation of cells, a fundamental event in embryonic development, is dysregulated in pathological conditions such as tumorigenesis and angiogenesis. Such differentiation is affected by cell-cell as well as cell-matrix adhesion molecules, which are in turn controlled by extracellular factors (*1*). These processes are regulated by factors that act through G protein–coupled receptors (GPRs) and receptor tyrosine kinases (*2*), as well as by bioactive lipids such as lysophosphatidic acid (LPA), SPP, and sphingosylphosphorylcholine (*3*). Signaling pathways regulated by the small guanine nucleotide binding protein Rho participate in the formation of cadherin-dependent cell-cell contacts (*4*).

The *EDG-1* transcript was cloned as an immediate-early gene induced during differentiation of human endothelial cells into capillary-like tubules, an in vitro model of angiogenesis (*5*). The EDG-1 polypeptide is a prototypical member of an orphan receptor subfamily composed of EDG-2/vzg-1, ARG16/H218, and EDG-3 (*6*, *7*). However, the ligands, physiologically relevant signaling pathways, and function of EDG-1 have not yet been described. We transfected human embryonic kidney 293 fibroblasts (HEK293), which do not express the EDG-1 receptor (*8*), with FLAG epitope–tagged human *EDG-1* cDNA in the *pCDNANeo* expression vector. Several clones expressing EDG-1 (HEK293*EDG-1*) or transfected with vector alone (HEK-293*pCDNA*) were isolated (*9*). All *EDG-1*–expressing clones grown in the presence of fetal bovine serum (FBS) formed a network of cell-cell aggregates (Fig. 1). This morphology resembles the network formation of differentiated endothelial cells. In contrast, vector-transfected cells were evenly distributed and maintained a normal, fibroblast-like morphology. Morphogenesis of HEK293*EDG-1* cells was suppressed by incubation with an antibody that binds to the NH_2-terminal FLAG epitope on the EDG-1 receptor (anti-M2) (Fig. 1). We characterized the factor in serum that synergizes with EDG-1 to induce morphological changes. Heat treatment (95°C, 1 hour) of FBS did not inhibit its effect on morphogenesis of *EDG-1*–transfected cells. However, removal of lipids from FBS by charcoal stripping or by butan-1-ol extraction (*10*) completely removed the factor that caused morphogenesis. Furthermore, the lipids extracted from the charcoal (*10*) induced morphogenetic differentiation in HEK293-*EDG-1* cells (Fig. 1). Thus, the EDG-1 ligand is present in the lipid fraction of serum.

Of serum-borne lipids, only SPP induced EDG-1–dependent morphogenesis, where-

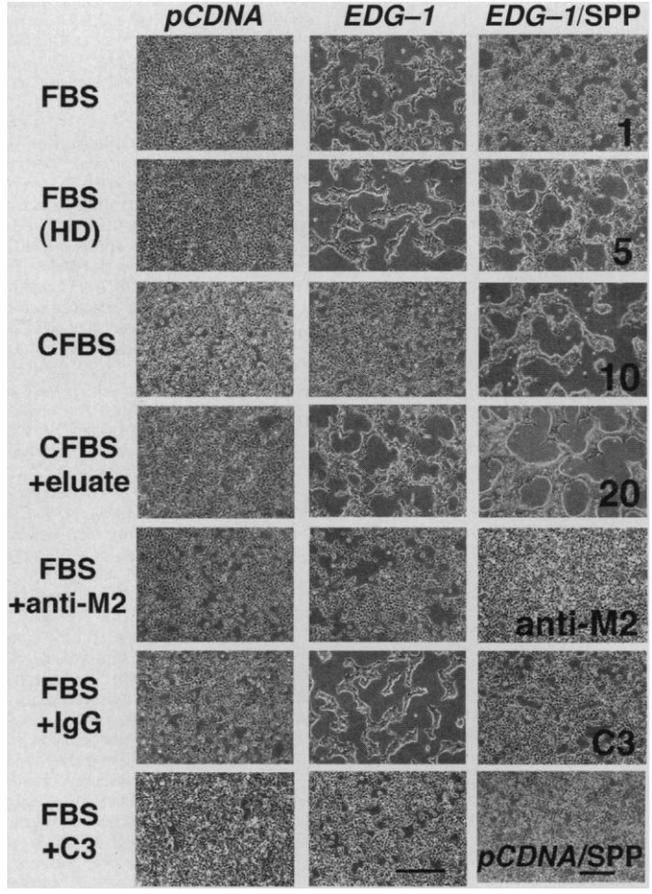

Fig. 1. Presence of a ligand for EDG-1 in the lipid fraction of serum. (**Left**) HEK293*pCDNA* (*pCDNA*) and (**Middle**) HEK293*EDG-1* (*EDG-1*) cells (7.5×10^5 cells/ml) were cultured in six-well plates in Dulbecco's minimum essential medium and 10 mM Hepes (pH 7.4) with the indicated supplements, and cellular morphology was examined after 12 to 15 hours. FBS, 10%; FBS (HD), FBS incubated at 95°C for 1 hour (10%); CFBS, 10%; eluate, serum-derived charcoal-bound lipid eluate (about 120 nmol phospholipid); anti-M2, antibody to FLAG (50 μg/ml; Eastman Kodak); IgG, irrelevant mouse immunoglobulin G (50 μg/ml); C3, C3 exotoxin [cells were pretreated with exotoxin (10 μg/ml) for 2 days; Calbiochem]. Scale bar, 94 μm. (**Right**) EDG-1–dependent morphogenetic differentiation induced by SPP (*EDG-1*/SPP). HEK293-*EDG-1* cells were cultured as described above in medium containing 10% CFBS, and the indicated concentrations of SPP (1, 5, 10, or 20 μM) were added. HEK293*EDG-1* cells were treated with anti-M2 or C3 exotoxin in the presence of 20 μM SPP. *pCDNA*/SPP indicates the effect of 20 μM SPP on HEK293*pCDNA* cells. Scale bar, 56 μm.

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as sphingosine, sphingomyelin, ceramide, ceramide-1–phosphate, lysophosphatidyl serine, lysophosphatidyl ethanolamine, lysophosphatidyl inositol, lysophosphatidyl choline, leukotriene B4 and C4, platelet-activating factor, anandamide, 12-hydroxyeicosatetraenoic acid (HETE), 15-HETE, and 13-hydroxydodecanoic acid, at concentrations as high as 50 μM, were ineffective. LPA, a bioactive lipid structurally related to SPP, induced morphogenetic differentiation weakly at 20 to 50 μM (*11*). SPP induced morphogenesis at low doses (1 to 20 μM) (Fig. 1). EDG-1 is known to activate the mitogen-activated protein (MAP) kinase known as extracellular signal-regulated kinase 2 (ERK-2) through pertussis toxin (PTx)–sensitive G_i protein (*12*). However, PD98059 and PTx, which inhibit the ERK-2 signaling pathway and trimeric G_i proteins (*13*), respectively, did not inhibit EDG-1–mediated morphogenesis (*8*). However, C3 exotoxin, an inhibitor of Rho (*14*), completely prevented this morphogenesis, suggesting a requirement for Rho (Fig. 1).

HEK293*EDG-1* cells aggregated strongly in suspension, whereas HEK293*pCDNA* cells did not (Fig. 2A). This aggregation was Ca^{2+}-dependent and was completely prevented by EGTA. Incubation with the integrin antagonist RGD peptide did not affect cell-cell aggregation, indicating the lack of involvement of integrins. Cytochalasin B, an inhibitor of microfilaments and nonspecific aggregation of cells, also did not affect cell-cell aggregation. Because the cadherins mediate calcium-dependent homotypic adhesion mechanisms (*15*), we analyzed the amounts of cadherin family polypeptides in HEK293*EDG-1* and HEK293*pCDNA* cells. Expression of both P- and E-cadherins was increased in HEK293*EDG-1* cells (Fig. 2B). However, expression of cytoplasmic cadherin-associated proteins, such as α-, β-, and γ-catenin (*15*), was not altered. Moreover, the expression of focal adhesion kinase and paxillin, which are involved in the formation of focal adhesion complexes (*2*), was also unaltered. The expression of vascular endothelial cadherin (VE-cadherin or cadherin-5) was not observed in either vector- or *EDG-1*–transfected HEK293 cells (*8*). HEK293*EDG-1* cells had abundant well-developed adherens junction–like structures (Fig. 2C). Moreover, consistent with the morphogenetic differentiation, expression of P-cadherin in HEK293*EDG-1* cells was enhanced by FBS and SPP and was blocked in both cases by C3 exotoxin. However, inhibition of the G_i pathway with PTx did not inhibit the EDG-1–induced P-cadherin expression (Fig. 2D). Together, these data suggest that SPP signals through EDG-1 to regulate the biogenesis or the maintenance of the adherens junctional complexes and morphogenetic differentiation. Rho was required for both EDG-1–induced cadherin expression and formation of adherens junctions, consistent with the observation that the small guanosine triphosphatases (GTPases) Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts (*4*).

To provide further evidence that SPP is a ligand for EDG-1, we developed a radioligand binding assay. Specific ^{32}P-labeled SPP binding was time-dependent and was observed only in HEK293*EDG-1* cells, whereas binding was negligible to vector-transfected cells (Fig. 3A). SPP binding to HEK293*EDG-1* was saturable, and Scatchard analysis indicated a dissociation constant (K_d) of 8.1 nM and a maximum binding capacity of 661 fmol per 10^5 cells (Fig. 3B). Specific binding of [^{32}P]SPP experi-

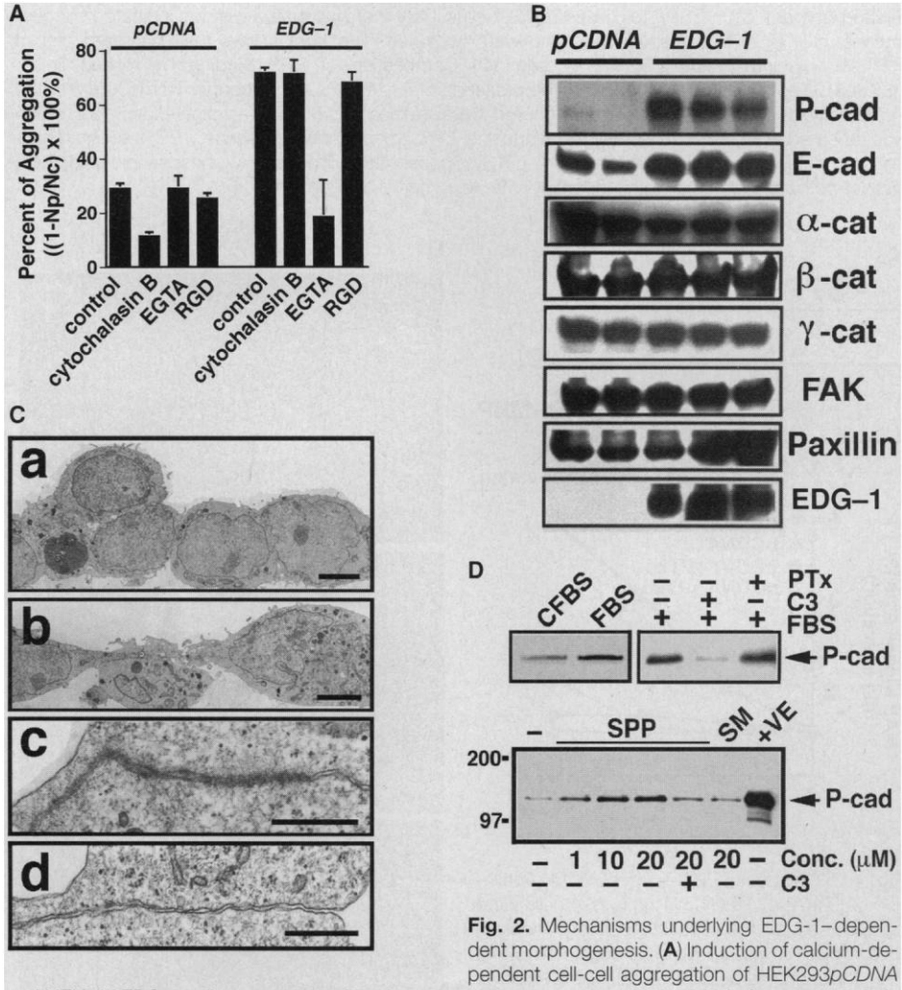

Fig. 2. Mechanisms underlying EDG-1–dependent morphogenesis. (**A**) Induction of calcium-dependent cell-cell aggregation of HEK293*pCDNA* and HEK293*EDG-1* cells was analyzed by the aggregation assay as described (*25*). Cytochalasin B (2 μM), EGTA (5 mM), or RGD peptide (1 mg/ml) was added to the medium before the initiation of the assay. Data represent mean ± SD of triplicate determinations from a typical experiment that was repeated two times. (**B**) Expression of P- and E-cadherin polypeptides. Cell extracts from two HEK293*pCDNA* and three HEK293*EDG-1* independently isolated clones were immunoblotted with various antibodies (Transduction Laboratories, Lexington, Kentucky) or with anti-M2; cad, cadherin; cat, catenin; FAK, focal adhesion kinase. (**C**) Formation of adherens junctions. Transmission electron micrographs of thin sections of HEK293*EDG-1* (a and c) and HEK293*pCDNA* (b and d) cells cultured in the presence of FBS. (a and b) Note the aggregated, clustered nature of HEK293*EDG-1* cells. Scale bar, 5 μm. (c and d) Detail of a representative cell-cell junction from both cell types. Scale bar, 0.5 μm. (**D**) Ligand- and Rho-dependent expression of P-cadherin. HEK293*EDG-1* cells were cultured in FBS (10%), CFBS (10%), or FBS (10%) containing C3 exotoxin (10 μg/ml) or PTx (100 ng/ml) for 3 days (top) or were treated with the indicated concentrations (Conc.) of lipids with or without the C3 exotoxin for 3 days in medium containing CFBS (10%) (bottom). Cell extracts were analyzed for P-cadherin expression by immunoblot analysis. Data are from a representative experiment that was repeated at least two times. SM, sphingomyelin; +VE, positive control P-cadherin protein from A431 cell extracts. The numbers to the left are molecular weight markers.

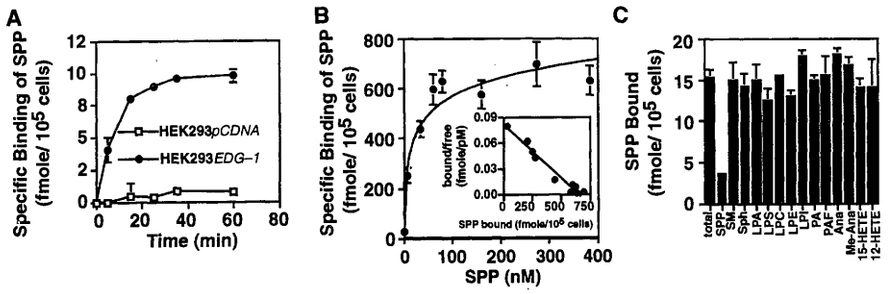

Fig. 3. Binding of SPP to EDG-1 (*26*). (**A**) Time dependence of specific [^{32}P]SPP binding. Cells were incubated with 1 nM [^{32}P]SPP for the indicated times, and specific binding was determined (*26*). (**B**) Binding isotherm of [^{32}P]SPP to HEK293*EDG-1* cells. Cells were incubated with the indicated concentrations of [^{32}P]SPP, and specific binding was measured. The inset shows the Scatchard plot of [^{32}P]SPP binding to HEK293*EDG-1* cells. (**C**) Competition of SPP binding by related lipids. HEK293*EDG-1* cells were incubated in the presence of 1 nM [^{32}P]SPP without or with 100 nM of the indicated lipids, and total binding was measured. Data are means ± SD from a typical experiment, which was repeated at least two times. Sph, sphingosine; LPS, lysophosphatidyl serine; LPC, lysophosphatidyl choline; LPE, lysophosphatidyl ethanol; LPI, lysophosphatidyl inositol; PA, phosphatidic acid; PAF, platelet-activating factor; Ana, anandamide; Me-Ana, methyl anandamide.

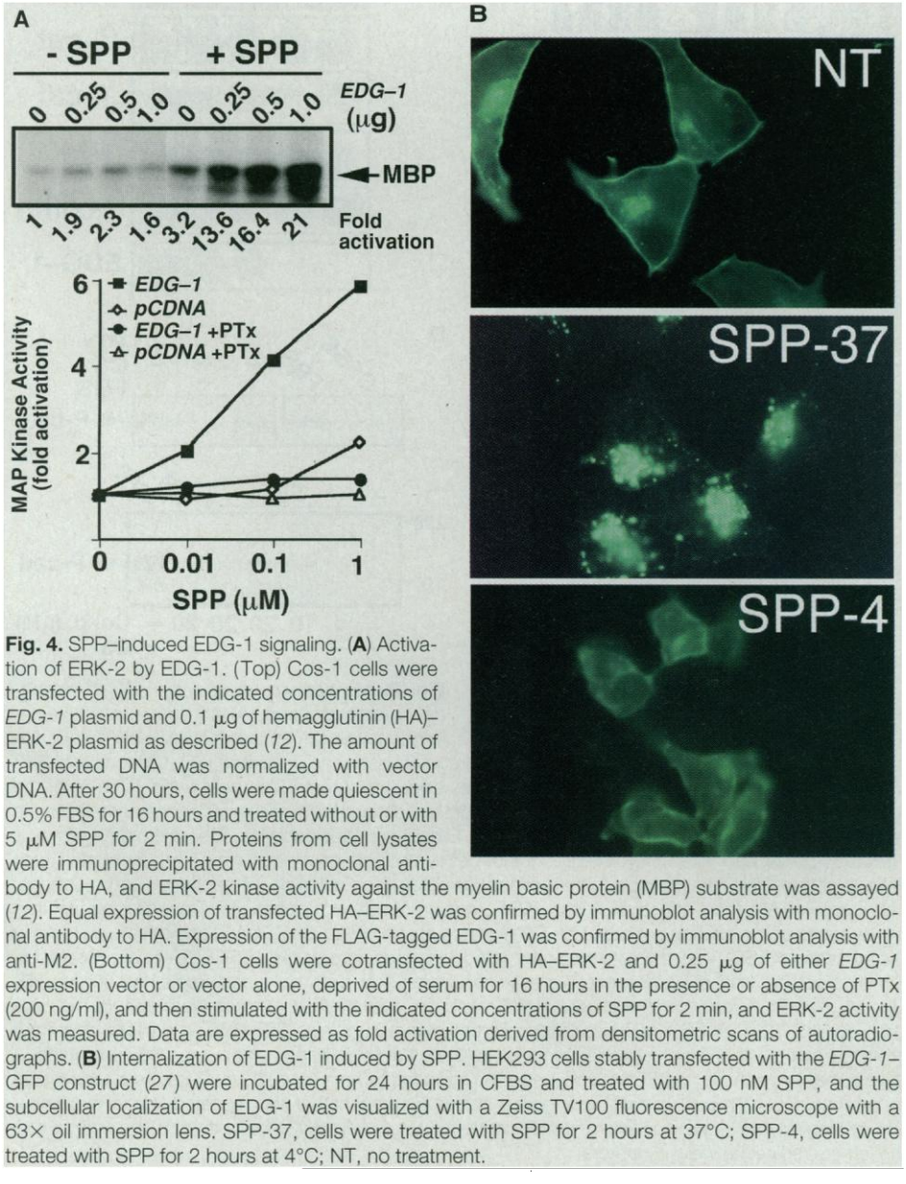

Fig. 4. SPP–induced EDG-1 signaling. (**A**) Activation of ERK-2 by EDG-1. (Top) Cos-1 cells were transfected with the indicated concentrations of *EDG-1* plasmid and 0.1 μg of hemagglutinin (HA)–ERK-2 plasmid as described (*12*). The amount of transfected DNA was normalized with vector DNA. After 30 hours, cells were made quiescent in 0.5% FBS for 16 hours and treated without or with 5 μM SPP for 2 min. Proteins from cell lysates were immunoprecipitated with monoclonal antibody to HA, and ERK-2 kinase activity against the myelin basic protein (MBP) substrate was assayed (*12*). Equal expression of transfected HA–ERK-2 was confirmed by immunoblot analysis with monoclonal antibody to HA. Expression of the FLAG-tagged EDG-1 was confirmed by immunoblot analysis with anti-M2. (Bottom) Cos-1 cells were cotransfected with HA–ERK-2 and 0.25 μg of either *EDG-1* expression vector or vector alone, deprived of serum for 16 hours in the presence or absence of PTx (200 ng/ml), and then stimulated with the indicated concentrations of SPP for 2 min, and ERK-2 activity was measured. Data are expressed as fold activation derived from densitometric scans of autoradiographs. (**B**) Internalization of EDG-1 induced by SPP. HEK293 cells stably transfected with the *EDG-1*–GFP construct (*27*) were incubated for 24 hours in CFBS and treated with 100 nM SPP, and the subcellular localization of EDG-1 was visualized with a Zeiss TV100 fluorescence microscope with a 63× oil immersion lens. SPP-37, cells were treated with SPP for 2 hours at 37°C; SPP-4, cells were treated with SPP for 2 hours at 4°C; NT, no treatment.

enced competition only from unlabeled SPP and not from other lipids that did not induce morphogenetic differentiation (Fig. 3C). Thus, binding of SPP to EDG-1 is of high affinity and high specificity, consistent with the possibility that SPP is a physiological ligand for EDG-1. SPP is present in serum (*16*), where it occurs at concentrations greater than the measured K_d for EDG-1. LPA, another serum-borne lysolipid, signals through the related EDG-2/vzg-1 receptor to regulate cell rounding and serum response factor–dependent transcription (*7*, *17*).

If SPP is a physiological ligand for EDG-1, it should activate EDG-1–regulated signaling pathways. Transfection of cells with *EDG-1* causes G_i-dependent activation of ERK-2 (*12*), and SPP activates ERK-1 and ERK-2 in various cells (*18*). Stimulation of ERK-2 activity by nanomolar concentrations of SPP was potentiated by expression of EDG-1, and this effect was blocked in cells treated with PTx (Fig. 4A). Thus, activation of EDG-1 by SPP transduces two distinct intracellular signaling pathways. First, the G_i protein–coupled ERK-2 pathway is activated. Previous studies have indicated that the βγ subunit of the heterotrimeric G protein activates the small GTPase Ras, which in turn stimulates the ERK pathway (*19*). Second, Rho-coupled pathways that regulate morphogenesis are activated. Activation of the Rho pathway by GPRs is mediated by the G_{12}/G_{13} family of heterotrimeric G proteins (*20*); however, the intermediate signaling steps are poorly understood. Indeed, similar activation of both pathways has been shown for LPA, a related bioactive lipid (*3*, *4*).

Ligand binding to GPRs induces internalization of receptors (*21*). To examine the cellular localization of EDG-1, we expressed the EDG-1 receptor fused with a COOH-terminal green fluorescent protein (GFP). The EDG-1–GFP polypeptide was localized primarily on the plasma membrane (Fig. 4B). Treatment of cells with 100 nM SPP for 2 hours at 37°C caused translocation of EDG-1 into intracellular vesicles. Neither incubation with SPP at 4°C nor incubation with other lipids that do not compete for high-affinity SPP binding to EDG-1 induced receptor trafficking into intracellular vesicles. Moreover, localization of the GFP control polypeptide in the cytosol was not altered by SPP treatment.

SPP, stored in platelets and released by platelet activation, is now recognized as a potent bioactive lipid with multiple biological activities (*22*). Our results reveal a role for SPP as an extracellular ligand for the endothelial-derived receptor EDG-1

that regulates morphogenetic differentiation. Because serum concentrations of SPP are estimated to be 60 times greater than the K_d for binding to EDG-1 (*16*), our data argue that SPP may be a physiologically relevant ligand for EDG-1.

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9. HEK293 fibroblasts were transfected with FLAG epitope–tagged human *EDG-1* cDNA in the *pCDNA-Neo* expression vector as described (*12*). Stably transfected cells were subcloned twice to isolate pure clones. Expression of FLAG-tagged *EDG-1* in transfected clones was verified by immunoblot analysis. Three such clones (HEK293*EDG-1*) and two clones obtained in a similar manner with the vector (HEK293*pCDNA*) were used in the present study.
10. Charcoal-stripped FBS (CFBS) was prepared by incubation of 10 ml of FBS with 1 g of activated charcoal (Sigma) at 4°C overnight. After centrifugation (2000*g* for 10 min), the supernatant was filtered through a 0.22-μm cellulose acetate filter (Corning). Charcoal-bound lipids were eluted with 1 M acetic acid and methanol (1:1). The eluate was vacuum-dried and briefly sonicated in fatty acid–free bovine serum albumin (BSA) (4 mg/ml; Sigma). FBS was extracted three times with 50% (v/v) of butan-1-ol to remove serum-borne lipids.
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25. Aggregation assays were done essentially as described (*23*), with minor modifications. Single-cell suspensions were plated into dishes coated with 1% agarose and rotated at 80 revolutions per minute for 90 min at 37°C to allow aggregation. Quantitative analysis of cell aggregation was performed with a Coulter (Miami, FL) Particle Counter with an aperture size of 100 μm. The extent of cell aggregation is represented by the index $(1 - N_o/N_c) \times 100\%$, where N_o and N_c are the total number of particles counted and the total number of cells used, respectively.
26. [^{32}P]SPP was synthesized enzymatically with partially purified sphingosine kinase as described previously (*24*). HEK293*pCDNA* or HEK293*EDG-1* cells (10^5) were incubated at 4°C in 200 μl of binding buffer [20 mM tris-HCl (pH 7.4), 100 mM NaCl, 15 mM NaF, 2 mM deoxypyridoxine, 0.2 mM phenylmethylsulfonyl fluoride, aprotinin (1 μg/ml), leupeptin (1 μg/ml), and fatty acid–free BSA (4 mg/ml)], with the indicated concentration of [^{32}P]SPP for 30 min in the absence or presence of unlabeled lipid competitors. Cells were pelleted at low speed in a microcentrifuge and washed twice with binding buffer containing fatty acid–free BSA (0.4 mg/ml). Bound [^{32}P]SPP was quantitated by scintillation counting. Specific binding was calculated as total binding minus binding in the presence of 100-fold excess of unlabeled SPP.
27. The enhanced GFP cDNA (Clontech, Palo Alto, CA) was fused at the COOH-terminus of the *EDG-1* cDNA, and the chimera was cloned into a eukaryotic expression vector containing a cytomegalovirus promoter (*pEGFP*). Expression of the correct polypeptide was confirmed by DNA sequencing as well as by immunoblot analysis of transfected cells. HEK293 clones stably expressing EDG-1–GFP were isolated by standard procedures (*12*).

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