RESEARCH COMMUNICATION

Sphingosine 1-phosphate signalling through the G-protein-coupled receptor Edg-1

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Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are structurally related lipid mediators that act on distinct G-protein-coupled receptors to evoke similar responses, including Ca^{2+} mobilization, adenylate cyclase inhibition, and mitogen-activated protein (MAP) kinase activation. However, little is still known about the respective receptors. A recently cloned putative LPA receptor (Vzg-1/Edg-2) is similar to an orphan G_i -coupled receptor termed Edg-1. Here we show that expression of

Edg-1 in Sf9 and COS-7 cells results in inhibition of adenylate cyclase and activation of MAP kinase (G_i -mediated), but not Ca^{2+} mobilization, in response to S1P. These responses are specific in that (i) S1P action is not mimicked by LPA, and (ii) Vzg-1/Edg-2 cannot substitute for Edg-1. Thus the Edg-1 receptor is capable of mediating a subset of the cellular responses to S1P.

INTRODUCTION

Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are platelet-derived lipid mediators that act on their cognate G-protein-coupled receptors to evoke multiple cellular responses (for review see [1,2]). Typical receptor-mediated responses to S1P and LPA include stimulation of phospholipase C (PLC) and consequent Ca^{2+} mobilization, inhibition of adenylate cyclase, mitogen-activated protein (MAP) kinase activation, mitogenesis and cytoskeletal changes such as cell rounding and neurite retraction [1–10]. Although S1P and LPA are structurally similar, there is compelling evidence that both agonists act through distinct receptors [9–12], possibly representing multiple subtypes [2,13]. Whether S1P may serve an additional role as intracellular second messenger, as originally proposed [14,15], remains a matter of debate [2].

While much effort has been focused on S1P- and LPA-induced signalling events, little is still known about the identity of the respective receptors. Recently, two putative LPA-receptor cDNAs have been isolated [16,17]. One receptor, cloned from *Xenopus*, enhances LPA-induced Cl^- currents in oocytes [16]; it shows remarkably little sequence similarity to other G-protein-coupled receptors. Another putative LPA receptor, termed Vzg-1, was cloned from mouse neuronal cells [17]. Its overexpression potentiates cell rounding and neurite retraction in response to LPA [17]. Since Vzg-1 is the mouse homologue of an earlier cloned ovine orphan receptor termed Edg-2 [18], we will refer to Vzg-1 as Edg-2. Recently, the human homologue of Edg-2 was shown to enhance LPA-induced reporter-gene transcription in HEK293 cells [19]. To date, a functional cell-surface receptor for S1P has not been identified.

Among the closest homologues of Edg-2/Vzg-1 are the cannabinoid receptor (30 % identity), whose natural ligand is a fatty acid derivative, and the orphan G-protein-coupled receptor Edg-1 (37 % identity) [20]. Edg-1 binds to $\text{G}_i\alpha$, and signals sustained G_i -mediated MAP kinase activation when overexpressed in HEK293 cells [21]. However, the identity of the ligand(s) for Edg-1 has been elusive. Since receptors of one

subfamily may bind the same or a related ligand, we set out to examine the signalling properties of Edg-1 towards S1P and LPA, using Edg-2 as a control. Our results indicate that Edg-1, but not Edg-2, is capable of mediating a subset of the cellular actions of S1P. These Edg-1-mediated responses are specific in that LPA fails to mimic S1P.

EXPERIMENTAL

Cell culture, transfection and antibodies

Sf9 insect cells were grown and infected as described in [22]. COS-7 cells were maintained in Dulbecco's modified Eagle medium supplemented with 8 % fetal-calf serum (FCS) and antibiotics. COS-7 cells were transfected using the DEAE-dextran method and assayed 2 days after transfection. Antibodies 9E10, P5D4 and M2 (Eastman Kodak Company, New Haven, CT, U.S.A.) were used to detect expression of the Myc-, VSV (vesicular-stomatitis virus)- and FLAG-tag respectively.

cDNA cloning and plasmid construction

The human Edg-1 cDNA [19] was kindly provided by T. Hla (Department of Physiology, University of Connecticut, Farmington, CT, U.S.A.). To extend the Edg-1 cDNA with an in-frame C-terminal tag derived from the VSV glycoprotein (15 amino acids), the entire Edg-1 coding region was amplified using the primers 5'-CCACGGATCCACCACCATGGGGCCCACC-AGC-3', containing the Kozak consensus sequence, and 5'-GGTTGCGGCCGCCGAAGAAGAGTTGACGTTTC-3', containing a *NotI* site for subcloning. Amplified product was purified and subcloned into the pcDNA3 expression vector (Invitrogen Corp., San Diego, CA, U.S.A.) containing a C-terminal VSV-tag, yielding pcDNA3-*edg1*-VSV.

The complete coding region of Edg-2 [17,18] was amplified from a mouse brain cDNA library using the primers 5'-CAC-AGAATTCGCCACCATGGCAGCTGCCTCTACTTCC-3', containing the Kozak consensus site, and 5'-CACTGCGGC-

Abbreviations used: EGF, epidermal growth factor; FCS, fetal-calf serum; LPA, lysophosphatidic acid; MAP, mitogen-activated protein; MBP, myelin basic protein; NKA, neurokinin A; PLC, phospholipase C; PTX, pertussis toxin; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphocholine; Indo-1 AM, Indo-1 acetoxymethyl ester; VSV, vesicular-stomatitis virus.

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CGCCAACCACAGAGTGGTCGTTGCTG-3', containing a *NotI* cloning site to facilitate C-terminal tagging. PCR products were subcloned into pBluescript, verified by sequencing and introduced into the pcDNA3 vector containing a C-terminal VSV-tag. The resulting expression construct was named pcDNA3-*edg2*-VSV.

Generation of recombinant baculoviruses

Recombinant baculovirus encoding the human NK-2 receptor has been characterized previously [23]. For generation of recombinant Edg-1 and Edg-2 viruses, the Bac-to-Bac expression system (Life Technologies Inc., Breda, The Netherlands) was used. Briefly, the entire coding regions of Edg-1 cDNA containing an N-terminal FLAG-tag and Edg-2 cDNA containing a C-terminal VSV-tag were subcloned in pFASTBAC (Life Technologies, Inc.). Recombinant baculoviral DNA was selected according to the manufacturer's instructions and transfected into Sf9 cells. Recombinant baculovirus obtained from the culture supernatant was then amplified in several rounds. To test recombinant protein expression, infected Sf9 cells were lysed in Nonidet P40 lysis buffer [20 mM Tris/HCl (pH 7.5)/100 mM NaCl/5 mM EDTA/1% Nonidet P40/protease inhibitors]. Lysates were cleared by centrifugation and mixed with 2×SDS sample buffer. Protein samples were separated by SDS/PAGE, transferred to nitrocellulose and probed with the appropriate antibodies. Signals were detected using the ECL (enhanced chemiluminescence) kit (Amersham International).

cAMP measurements

Sf9 cells were infected with recombinant baculoviruses and harvested 2 days after infection. Cells were washed once and then incubated in serum-free Grace's insect medium (Life Technologies Inc.) for 2 h. Cells were incubated with 5 μ M forskolin in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.5 mM) for 10 min followed by a 20 min stimulation with 2.5 μ M S1P or LPA. cAMP levels were determined using a [3 H]cAMP assay kit (Biotrak; Amersham International), following the instructions of the manufacturer.

Ca²⁺ measurements

Infected Sf9 cells were harvested 2 days after infection, gently washed in serum-free Grace's medium, and loaded with 5 μ M Indo-1 acetoxymethyl ester (Indo-1 AM; Molecular Probes Inc., Eugene, OR, U.S.A.) for 45 min. Free Indo-1 AM was washed out and cells were resuspended in HBS buffer [10 mM Hepes (pH 6.0)/140 mM NaCl/5 mM KCl/1 mM MgCl₂/1 mM CaCl₂/10 mM glucose] at a concentration of 10⁶ cells/ml. Agonist-induced Indo-1 fluorescence was monitored in quartz cuvettes kept at 26 °C as described in [4].

MAP kinase activation assays

MAP kinase activation was analysed by either the mobility-shift assay or by *in vitro* myelin-basic-protein (MBP)-phosphorylation assays. In both cases, COS cells were co-transfected in six-well plates with 200 ng of pEXV-Erk2-Myc and 500 ng of pcDNA3 encoding VSV-tagged Edg-1 or Edg-2. At 2 days after transfection, cells were serum starved for 4 h and stimulated as indicated. For mobility-shift assays, cells were directly lysed in SDS sample buffer and total cell lysates separated on SDS/10% -PAGE gels. Separated proteins were transferred to nitrocellulose and probed with anti-Myc antibody 9E10 using standard Western-blotting procedures. Blots were developed using the

ECL system. *In vitro* MBP phosphorylations were performed on 9E10-immunoprecipitated Erk2 as described in [6]. Where indicated, cells were pretreated overnight with 200 ng/ml pertussis toxin (PTX).

RESULTS AND DISCUSSION

We expressed Edg-1 (VSV epitope-tagged) in both insect Sf9 cells and mammalian COS-7 cells to measure three distinct G-protein-mediated responses to S1P and LPA, notably (i) inhibition of cAMP accumulation, (ii) stimulation of PLC, as monitored by Ca²⁺ mobilization, and (iii) activation of MAP kinase. The related putative LPA receptor, Edg-2, served as a control.

Inhibition of adenylate cyclase

Both LPA and S1P are known to inhibit adenylate cyclase [3,10], a classical G_i-mediated signalling event. To test whether Edg-1 couples to inhibition of adenylate cyclase in response to S1P or LPA, we used the baculovirus/Sf9-cell expression system. In a previous study, we used Sf9 cells as a convenient system for studying the signalling properties of the human NK-2 receptor for neurokinin A (NKA; [23]). Moreover, initial pilot studies revealed that Sf9 cells are unresponsive to both LPA and S1P. At 48 h after infection with recombinant baculovirus, Sf9 cells expressing Edg-1 or Edg-2 were treated with forskolin to raise intracellular cAMP levels. Figure 1(A) illustrates that Sf9 cells

Figure 1 Inhibition of cAMP accumulation

(A) Sf9 cells expressing either Edg-1 or Edg-2 were treated with forskolin (FK, 5 μ M) to raise cAMP levels. LPA and S1P (2.5 μ M) were added and, after 20 min, cellular cAMP content was determined as described in the Experimental section. C, non-treated control cells. Data are means for two separate experiments, with the highest and lowest of both values indicated by bars. (B) Expression levels of Edg-1 (FLAG-tagged) and Edg-2 (VSV epitope-tagged) as determined by immunoblotting of total Sf9 cell lysates. Control lanes represent non-infected Sf9 cells. The predicted molecular masses of Edg-1 and Edg-2 are about 40 kDa. Higher-molecular-mass bands represent receptor aggregates.

Figure 2 Ca^{2+} mobilization

Sf9 cells expressing Edg-1, Edg-2 or the NK-2 receptor for neurokinin A (NKA) were loaded with the fluorescent Ca^{2+} indicator Indo-1 and stimulated with the indicated agonists, followed by addition of ionomycin (iono). F, Indo 1 fluorescence. Final concentrations: LPA, $5 \mu\text{M}$; S1P, $0.5 \mu\text{M}$; NKA, $1 \mu\text{M}$; ionomycin, $5 \mu\text{M}$. Control, addition of medium ($5 \mu\text{l}$). For receptor expression controls, see Figure 1.

Figure 3 Activation of MAP kinase

COS-7 cells were transfected with empty vector (control), Edg-1 or Edg-2 (VSV epitope-tagged) together with Myc-tagged Erk2. Cells were stimulated with the indicated agonists for 5 min and lysed. Cell lysates were analysed by SDS/PAGE followed by immunoblotting using anti-Myc antibody 9E10 (left panels). The phosphorylated, activated forms of Erk2 display reduced electrophoretic mobility. Right panel: expression controls of Edg-1/Edg-2 receptors by immunoblotting using anti-VSV antibody. Final concentrations: BSA, $1 \mu\text{M}$; LPA, S1P and SPC, $1 \mu\text{M}$ (in the presence of $1 \mu\text{M}$ BSA); C, medium control ($5 \mu\text{l}$). *edg-1* and *-2* refer to transfected cDNA.

expressing Edg-1, but not those expressing Edg-2, show an inhibition of forskolin-induced cAMP accumulation following addition of S1P. Figure 1(A) also shows that Edg-1 does not mediate such an effect in response to LPA. Receptor expression

controls are shown in Figure 1(B). From these results we conclude that Edg-1, but not Edg-2, mediates S1P-induced inhibition of adenylate cyclase.

Ca^{2+} mobilization

Since S1P and LPA can activate PLC, resulting in Ca^{2+} mobilization from internal stores, we examined Ca^{2+} signalling in Edg-1/Edg-2-expressing Sf9 cells treated with either S1P or LPA. The G_i -PLC-coupled NK-2 receptor was used as a control [23]. At 48 h after infection with recombinant baculovirus, cells were loaded with the fluorescent Ca^{2+} indicator Indo-1, and agonist-induced changes in cytosolic Ca^{2+} concentration were measured in both Ca^{2+} -containing and Ca^{2+} -free medium. Under conditions where the liganded NK-2 receptor mediates significant Ca^{2+} mobilization from internal stores, indicative of PLC activation, Edg-1 failed to transmit a detectable Ca^{2+} signal in response to either S1P or LPA (Figure 2). Similarly, Edg-2 did not mediate a detectable Ca^{2+} signal (Figure 2). Also, when expressed in COS-7 cells (see below), Edg-1 failed to mediate S1P-induced Ca^{2+} mobilization (results not shown). Taken together, these results indicate that neither Edg-1 nor Edg-2 couples to stimulation of PLC in response to S1P or LPA.

MAP kinase activation

To determine receptor-mediated MAP kinase activation, we used the COS-7 cell expression system. In COS cells, as in fibroblasts, G-protein-linked receptor agonists activate MAP kinase through the G_i -Ras pathway [3,6,24–26]. Our initial MAP kinase activity measurements revealed that COS-7 cells show hardly any or no response to S1P, while they do show a marked response to LPA. We measured receptor-mediated activation of the p42 MAP kinase (Erk2) in transfected COS-7 cells by two methods. First, we monitored the electrophoretic mobility shift of Erk2 that reflects its phosphorylation and activation. Figure 3 shows agonist-induced Erk2 mobility shifts in COS-7 cells expressing either Edg-1 or Edg-2, as compared with COS-7 cells transfected with empty vector. It is seen that Edg-1-expressing cells show S1P-induced MAP kinase activation, whereas the response to LPA is not affected. Figure 3 also shows that the Erk2 shift in response to LPA or sphingosylphosphocholine (SPC), which acts through a distinct G-protein-coupled receptor [2,27,28], is not enhanced following Edg-1 expression. Furthermore, COS-7 cells that overexpress Edg-2 do not respond to S1P, nor do they show an enhanced response to LPA or SPC (Figure 3).

Secondly, to quantify the enhanced response to S1P, we performed immunocomplex Erk2 kinase assays MBP as an exogenous substrate. As shown in Figure 4(A), S1P treatment of Edg-1-expressing COS-7 cells results in enhanced Erk2 activity when compared with empty vector-expressing cells, in agreement with the Erk2 mobility-shift data of Figure 3. Again, no effect of S1P was observed in Edg-2-expressing cells, nor when LPA, SPC or epidermal growth factor (EGF) were used as agonists (Figure 4A). Figure 4(B) shows that Erk2 expression levels were equal in these experiments. Maximal MAP kinase activation responses were observed at $1 \mu\text{M}$ S1P, with half-maximal responses estimated at about 50 nM (results not shown). Pretreatment of the cells with PTX (200 ng/ml ; 16 h) resulted in almost complete inhibition of S1P-induced MAP kinase activation (Figure 4C), indicating a critical role for G_i . From these results we conclude that Edg-1, but not Edg-2, mediates S1P-induced activation of the G_i -MAP kinase pathway.

Figure 4 *In vitro* MAP kinase activity

(A) COS-7 cells, transfected with the same constructs as in Figure 3, were stimulated for 5 min with the indicated agonists. Erk2 was immunoprecipitated and its kinase activity assayed using MBP as a substrate. Values are normalized ('-fold stimulation') with respect to empty vector controls (pcDNA3) and BSA addition. For Edg-1 and Edg-2 expression controls, see Figure 3 (right panel). Final concentrations: BSA, 1 μ M; LPA, S1P and SPC, 1 μ M (in the presence of 1 μ M BSA); EGF, 50 ng/ml; FCS, 10% (v/v). Error bars denote S.D. Number of experiments (*n*): S1P, LPA and BSA values, *n* = 5 for Edg-1 and *n* = 3 for Edg-2; SPC, EGF and FCS values, *n* = 1. In a typical experiment, non-derivatized values (relative to BSA control) for empty-vector- or Edg-2-transfected cells are: LPA, 17.9-fold; S1P, 1.8-fold; SPC, 5.7-fold; EGF, 26.3-fold; FCS, 20.3-fold. (B) Erk2 protein expression controls for the results shown in (A). Erk2 was immunoprecipitated from COS-7 cell lysates and protein levels detected by immunoblot using anti-Myc antibody 9E10. IgG, denotes IgG heavy chain. Constructs used: C, pcDNA3 (empty vector); lanes 1–6: Erk2-Myc + pcDNA3; lanes 7–12: Erk2-Myc + pcDNA3-*edg-1*-VSV; lanes 13–18: Erk2-Myc + pcDNA3-*edg-2*-VSV. Note that Erk2 expression levels are comparable. (C) PTX-sensitivity of Edg-1-mediated Erk2 activation in response to S1P (fold stimulation with respect to BSA/empty vector). Experimental details are as for (A). Concentrations: PTX, 200 ng/ml (16 h pretreatment); BSA, 1 μ M; S1P, 1 μ M (in the presence of 1 μ M BSA).

Concluding remarks

In the present study we have shown that the Edg-1 receptor is capable of mediating a subset of early responses to S1P, notably

inhibition of adenylate cyclase and activation of the G_i -MAP kinase pathway, but not activation of the PLC- Ca^{2+} signalling pathway. These effects are specific in that (i) LPA has no effect, and (ii) Edg-2 cannot replace Edg-1. The failure of Edg-1 to couple to Ca^{2+} mobilization suggests the existence of at least two S1P receptor subtypes: Edg-1 for the G_i pathway and another, as-yet-unidentified receptor for PLC stimulation. It is of note that our study also shows that Edg-2, whose overexpression stimulates LPA-induced shape changes [17] and reporter-gene activity [19], does not induce or enhance LPA responsiveness in Sf9 and COS-7 cells respectively. This strongly suggests that Edg-2 is not a G_q - and/or G_i -coupled LPA receptor; however, it remains possible that Edg-2 specifically couples to the RhoA GTPase to mediate LPA-induced morphological changes and early gene transcription. In this respect it will be interesting to examine whether other Edg subfamily members, notably AGR16/H218 [29,30] and Edg-3 [31] (which are ~50% homologous with Edg-1) may mediate certain actions of S1P, LPA or closely related bioactive lipids. Future ligand-binding studies should provide further evidence for S1P-receptor heterogeneity and reveal the respective ligand-binding affinities. However, quantitative binding studies with lysolipid agonists such as S1P and LPA are hampered by the lipophilic nature of the ligand, resulting in very high levels of non-specific binding (for example, see [32]). This limitation notwithstanding, the present study is the first to assign a cloned G-protein-coupled receptor to S1P action.

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