Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids

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Abstract The structural similarity of lysosphingolipids to lysophosphatidic acid (LPA) prompted a sequence-based search for lysosphingolipid receptors using cDNA sequence of the Edg2 human LPA receptor. Two closely related G protein-coupled receptors, rat H218 and human Edg3, are highly similar to Edg2. When overexpressed in Jurkat cells, H218 and Edg3 activated serum response element-driven transcriptional reporter gene in response to sphingosine 1-phosphate (S1P), dihydro-S1P and sphingosylphosphorylcholine, but not to LPA. H218 and Edg3 expressed in *Xenopus* oocytes conferred responsiveness to S1P and dihydro-S1P in agonist-triggered $^{45}Ca^{2+}$ efflux. Therefore, H218 and Edg3 are functional receptors for S1P and perhaps other closely related lysosphingolipids.

Key words: Lysosphingolipid; Sphingosine 1-phosphate; Sphingosylphosphorylcholine; G protein-coupled receptor; Rat H218; Human Edg3

1. Introduction

Several types of sphingolipids regulate cell growth, differentiation and programmed cell death as intracellular and extracellular messengers [1–3]. The lysosphingolipids, sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC), are potent mediators of fibroblast mitogenesis [4–6], platelet activation [7] and neurite retraction [8]. The biochemical signaling events elicited by S1P and SPC include intracellular Ca^{2+} mobilization [4,7,9–13], and activation of $I_{k(Ach)}$ [12,14] and MAP kinases [15]. Potential roles for lysosphingolipids in cellular growth and survival are also implied by their ability to oppose the effects of ceramide on cell proliferation and apoptosis [16,17].

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Abbreviations: S1P, sphingosine 1-phosphate; dh-S1P, dihydro-sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; SP, sphingosine; dh-SP, dihydro-sphingosine; LPA, lysophosphatidic acid; SRE, serum response element; G protein, guanine nucleotide-binding protein; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline

The nucleotide sequences reported in this paper has been submitted to the GenBank database with accession numbers AF022137, AF022138 and AF022139.

Early findings suggested that S1P was principally an intracellular second messenger mediating the proliferative effects of platelet-derived growth factor and evoking release of Ca^{2+} from internal stores by an inositol 1,4,5-trisphosphate (IP_3)-independent mechanism [18–20]. Recent studies showed that some signaling responses to S1P and SPC were blocked by pertussis toxin, suggesting that S1P and SPC might act via cell surface G protein-coupled receptors (GPCRs) [3,5,7,8,10–15]. A similar dependence on G proteins has been observed for responses to lysophosphatidic acid (LPA), which is structurally related to S1P and SPC. Two different GPCRs for LPA have recently been cloned and characterized by others and our laboratory [21–23]. However, the putative GPCRs for S1P and SPC have not been characterized at a molecular level.

We selected three orphan GPCRs with amino acid sequences highly related to that of the Edg2 LPA receptor to examine their ability to confer responsiveness to lysosphingolipids. Expression of the GPCRs H218 and Edg3, but not Edg1, conferred activation of an SRE-driven transcriptional reporter gene in Jurkat T cells and $^{45}Ca^{2+}$ efflux in *Xenopus* oocytes in response to S1P and related lysosphingolipids.

2. Materials and methods

2.1. Materials

Sphingosine 1-phosphate (S1P), sphingosylphosphorylcholine (SPC), sphingosine (SP), dihydro-sphingosine 1-phosphate (dh-S1P), dihydro-sphingosine (dh-SP) and C6 ceramide (C6) were obtained from Biomol (Plymouth, PA). 1-Oleoyl-lysophosphatidic acid (LPA), 1-β-D-galactosylsphingosine (psychosine, PS), fatty acid-free human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). The mammalian expression vectors pCDEF3 and pRC/CMV were obtained from Dr. Jerome Langer (UMD-New Jersey) and Invitrogen (La Jolla, CA), respectively. DNA polymerase *pfu* and PCRScript vector were from Stratagene (La Jolla, CA). DMRIE-C lipofection reagent for transfection and OPTI-MEM medium were from Gibco-BRL (Gaithersburg, MD). Jurkat leukemic T cells were obtained from Dr. Arthur Weiss (UCSF). Cell culture media and fetal bovine serum (FBS) were from UCSF Cell Culture Facilities. Plasmid pGL3-basic and luciferase assay reagents were from Promega (Madison, WI).

2.2. Cloning and plasmid constructs

The GenBank database was searched for sequences homologous to human Edg2 LPA receptor [23]. The recently recognized new subtype of human LPA receptor termed Edg4 [24] is the most homologous to Edg2 with 46% identity in amino acid sequence. The three 'orphan' GPCRs, human Edg1 [25], rat H218 [26] and human Edg3 (GenBank accession number X83864), are less homologous with 31–34% identity. The cDNAs encoding the open reading frames of these three 'orphan' GPCRs were amplified with *pfu* polymerase by RT-PCR using RNAs isolated from human lung, rat brain and human fetal brain, respec-

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tively. The primers used for PCR amplifications were 5′-GCTTCCTGGGGACACAGGGTTGGCACCATG and 5′-ACACTGGGGTGGCAGCGACCACCAAGT for *edg1*, 5′-GGCCAAGCTTGTGCTCAGTCCCATG and 5′-GCTGTCCCTCTAGACCCTTAATGGT for *h218*, 5′-TGTGAATTCCAAGTGATGGCAACTG and 5′-CGCATCTAGACGATCAGTTGCAGAAGATCCCATT for *edg3*. The amplified products were subcloned into expression vectors pCDEF3 (expression constructs designated Edg1/EF3, H218/EF3 and Edg3/EF3) and pRC/CMV (Edg1/CMV, H218/CMV and Edg3/CMV). The sequences of these expression plasmids were confirmed using an ABI automated DNA sequencer (HHMI DNA core facility, UCSF). The construction of a serum response element (SRE) luciferase reporter gene plasmid containing four copies of SRE (5′-AGGATGTCCATATTAGGACATCT) and a TATA box has been previously described [23,27].

2.3. Reporter gene assay

1×10^7 Jurkat T cells were co-transfected with 0.2 μg SRE reporter gene plasmid and 2 μg of either empty pCDEF3 vector, H218/EF3 or Edg3/EF3 using DMRIE-C reagent. After 4 h of transfection incubation in OPTI-MEM containing 10% FBS, cells were washed and starved in serum-free RPMI 1640 at 37°C for 8 h. Cells were then washed, resuspended in serum-free RPMI 1640 and transferred in 0.15 ml aliquots into 96-well plates. Various lipids to be tested were dissolved in serum-free RPMI 1640 containing 0.1 mg/ml of HSA and added to the cells followed by a 10 h incubation at 37°C. Cells were then disrupted by Reporter Lysis Buffer (Promega), and luciferase activities were measured using a Turner Designs 20/20 luminometer.

2.4. Oocyte expression and $^{45}Ca^{2+}$ efflux assay

Templates for in vitro transcription were prepared by linearizing Edg1/CMV, H218/CMV and Edg3/CMV with *Xba*I. 7-Methylguanosine-capped transcripts were synthesized using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) according to the manufacturer's instructions. Oocytes were surgically harvested from adult female *Xenopus laevis*, isolated by collagenase digestion, and microinjected with cRNA (25 ng/25 nl oocyte) [28,29]. After 24 h in culture to allow expression of the injected cRNAs, microinjected oocytes were radiolabeled with $^{45}Ca^{2+}$ and agonist-triggered $^{45}Ca^{2+}$ efflux was measured [28,29].

3. Results and discussion

We recently observed that S1P and SPC activated SRE-driven transcription via signaling pathways similar to those used by LPA receptors [24]. As lysosphingolipids and LPA are also similar in chemical structure, we hypothesized that their receptors might share close sequence homology. Accordingly, the sequence of the recently characterized human LPA receptor Edg2 [23] was used to search the GenBank database for related sequences. Most related to Edg2 was human Edg4, with 46% identity at the amino acid level. Edg4 was recently shown to be an LPA receptor analogous functionally to Edg2 [23,24]. Somewhat less homologous to Edg2 was a set of related orphan receptors: human Edg1 [25], rat H218 [26], and human Edg3. Edg1, H218, and Edg3 share 31–34% amino acid sequence identity with Edg2, but 45–60% identity with each other (Fig. 1). These receptors also share the unusual feature of lacking the cysteine in extracellular loop 1 common among most other GPCRs. These observations suggested that Edg1, H218, and Edg3 might form a subfamily of GPCRs related to, but distinct from, the LPA receptors.

To test the hypothesis that Edg1, H218, and Edg3 might be receptors for lysosphingolipids, we expressed these recombinant receptors in Jurkat cells and *Xenopus* oocytes and sought lysosphingolipid-induced responses. When co-transfected with the SRE-luciferase reporter gene in Jurkat cells, H218 and Edg3 mediated significant increases in SRE-driven luciferase expression in response to 1 μM SP, dh-SP, S1P, dh-S1P and

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                                                  TM1                          TM2
MATALPPRLQPVRGN -------ETLREHYQ YVGKLAGRLKEASEG STLTTVLFLVICSFI VLENLMVLIAIWKNN KFHNRMYFFIGNLAL
MGPTSVPLVKAHRSS VSDYVNYDIIVRHYN YTGKLNISADK-ENS IKLTSVVFILICCFI ILENIFVLLTIWKTK KFHRPMYYFIGNLAL
MGGLYSEYLNP---- -------EKVQEHYN Y-TKETLDMQE-TPS RKVASAFIIILCCAI VVENLLVLIAVARNS KFHSAMYLFLGNLAA
*                        **  *  *                    *  *   **  **        ***  ** * ****

                        TM3                                  TM4
CDLLAGIAYKVNILM SGKKTFSLSPTVWFL REGSMFVALGASTCS LLAIAIERHLTMIKM RPYDANKRHRVFLLI GMCWLIAFTLGALPI
SDLLAGVAYTANLLL SGATTYKLTPAQWFL REGSMFVALSASVFS LLAIAIERYITMLKM KLHNGSNNFRLFLLI SACWVISLILGGLPI
SDLLAGVAFVANTLL SGPVTLSLTPLQWFA REGSAFITLSASVFS LLAIAIERQVAIAKV KLYGSDKSCRMLMLI GASWLISL-LGGLPI
 ***** *   * *  **  *  * *  **  **** *  * **  * ********     *           *   **    * *   ** ***

                  TM5                                                    TM6
LGWNCLHNLPDCSTI LPLYSKKYIAFCISI FTAILVTIVILYARI YFLVKSSSRKVANHN N------SERSMALL RTVVIVVSVFIACWS
MGWNCISALSSCSTV LPLYHKHYILFCTTV FTLLLLSIVILYCRI YSLVRTRSRRLTFRK NISKASRSSENVALL KTVIIVLSVFIACWA
LGWNCLDHLEACSTV LPLYAKHYVLCVVTI FSVILLAIVALYVRI YFVVRSSHADVA--- -------GPQTLALL KTVTIVLGVFIICWL
 ****   *  ***  **** * *       *   *  ** ** ** *  *                      ***  ** **  *** **

                  TM7
PLFILFLIDVACRVQ ACPILFKAQWFIVLA VLNSAMNPVIYTLAS KEMRRAFFRL--VCN CLVRGRGARAS---- ---PIQPALDPSRSK
PLFILLLLDVGCKVK TCDILFRAEYFLVLA VLNSGTNPIIYTLTN KEMRRAFIR---IMS CCKCPSGDSAGK--- FKRPIIAGMEFSRSK
PAFSILLLDSTCPVR ACPVLYKAHYFFAFA TLNSLLNPVIYTWRS RDLRREVLRPLLCWR QGKGATGRRGGNPGH RLLPLRSSSSLERGL
* *   * *  * *   *  *  *  *   *  ***  ** ***      **   *            *           *        *


SSSSNNSSHSPKVK- ----EDLPHTDPSSC IMDKNAALQNGIFCN ---------------- --    378        human  Edg3
---SDNSSHPQKDE- ----GDNPETIMSSG NVNSSS--------- ---------------- --    381        human  Edg1
-HMPTSPTFLEGNTV VGEMTDLPSHRESSV GRDQVTSSCPSVPQV WRNPRLIGQVANGGT GA    397        rat    H218
                      * *    **
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Fig. 1. Alignment of amino acid sequences of human Edg3 (upper) with human Edg1 (middle) and rat H218 (lower) proteins. To achieve maximal homology, gaps (represented by hyphens) are introduced. Asterisks indicate identical residues conserved in all three proteins. The putative transmembrane domains are overlined and labeled as TM1 through TM7.

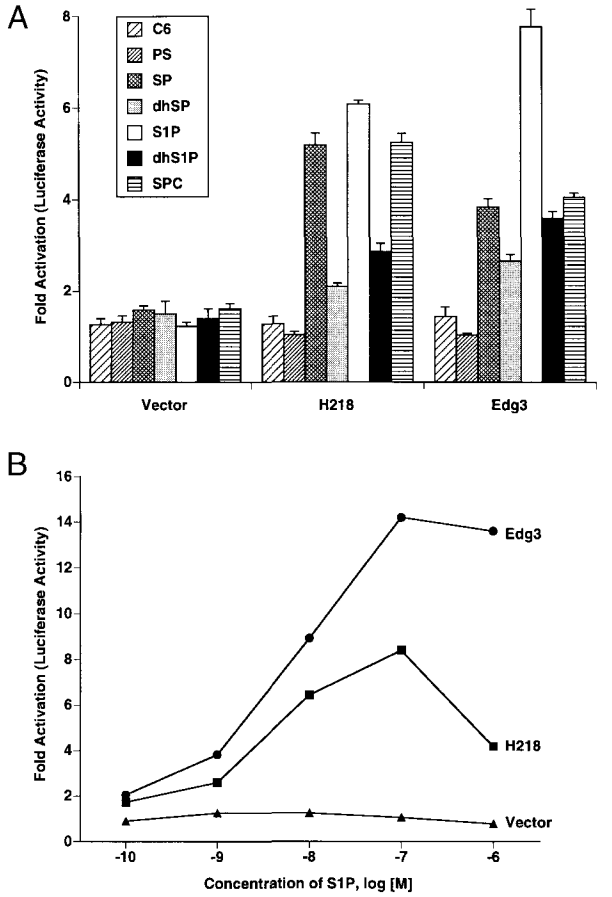

Fig. 2. Lysosphingolipid-induced activation of SRE-driven luciferase expression in transfected Jurkat T cells. A: Specificity of lysosphingolipid-induced responses. Jurkat cells co-transfected with SRE reporter gene plasmid and either empty pCDEF3 vector, H218/EF3 or Edg3/EF3 were treated with various lipids at 1 μM each. Data are plotted as fold increase over respective controls without agonist. Values are mean ± S.E.M. ($n=4$) of one representative experiment of three or more for each agonist. B: Concentration dependence of responses to S1P. Values are the average of duplicates from a representative of four different experiments.

SPC (Fig. 2A). The order of agonist activity at 1 μM was S1P > SPC > SP > dh-S1P > dh-SP for both H218- and Edg3-transfected cells. This rank order is similar to previous determinations of rank order of activity of these sphingolipids on fibroblast mitogenesis and AP-1 transcriptional activation [6]. Other structurally related lipids C6-ceramide, psychosine (Fig. 2A) and LPA (data not shown) failed to elicit significant responses. Jurkat cells transfected with Edg1/EF3 showed only marginal activation of 1.74 ± 0.07- and 1.68 ± 0.08-fold (mean ± S.E.M., $n=4$) in response to 1 μM S1P and SPC, respectively. Jurkat cells transfected with the control pCDEF3 vector did not show any significant increases in response to the lipids tested (Fig. 2A).

S1P-induced SRE activation was significant at 1 nM S1P, reached a maximum at 100 nM, and exhibited EC_{50} values of approximately 5 nM for H218 and 10 nM for Edg3 (Fig. 2B). SPC mimicked the effect of S1P, but with EC_{50} values one order of magnitude higher at approximately 50 nM and 100 nM for H218 and Edg3, respectively.

It was known that lysosphingolipids elicited increases in cytoplasmic Ca^{2+} via a G protein-mediated mechanism in a variety of mammalian cell types [3,7,11–13]. Attempts to evaluate the ability of transfected receptors to mediate Ca^{2+} signaling were confounded by signaling in the untransfected cells. These background responses are presumably mediated by endogenous lysosphingolipid receptors in many mammalian cells. We therefore expressed the candidate receptors in *Xenopus* oocytes and assessed lysosphingolipid-triggered $^{45}Ca^{2+}$ efflux, which reflects increases in cytoplasmic Ca^{2+} in these cells.

S1P and dh-S1P did not evoke responses in uninjected or control cRNA-injected oocytes (Fig. 3). By contrast, in oocytes injected with cRNA encoding H218 or Edg3, 1 μM S1P and dh-S1P evoked significant increases in $^{45}Ca^{2+}$ efflux (Fig. 3A). Similar to results of the SRE reporter assay, dh-S1P had a less potent effect than S1P in the $^{45}Ca^{2+}$ efflux assay. The structurally related lipids, ceramide, psychosine, SP and dh-SP, all at 1 μM, were without effect (Fig. 3A). Concentration-dependence studies of S1P showed that EC_{50} values were approximately 30 nM for both H218 and Edg3 receptors (Fig. 3B). Oocytes injected with cRNA encoding human Edg1 did not manifest significant increases in $^{45}Ca^{2+}$ efflux in response to S1P or dh-S1P (data not shown). Tests of SPC- and LPA-evoked signals were uninformative in the oocyte system due to inconsistent responses to SPC and high background response to LPA by uninjected oocytes, respectively.

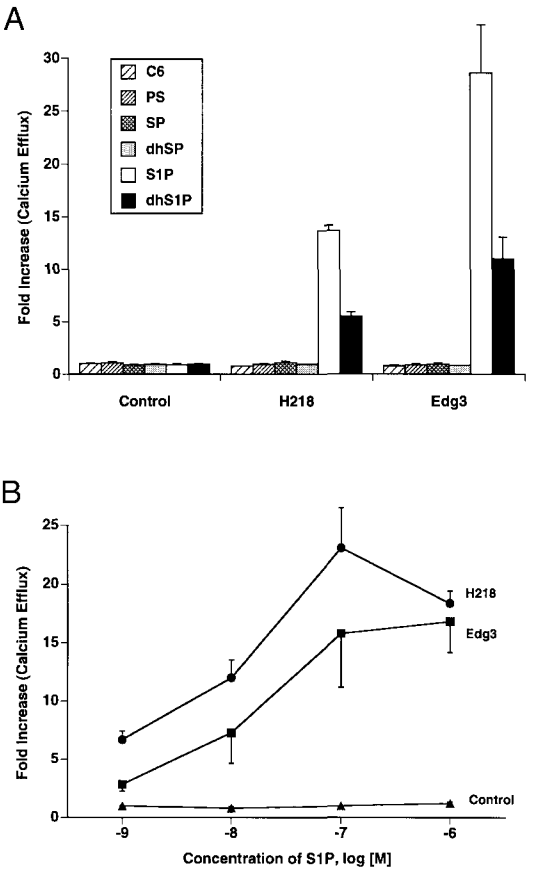

Fig. 3. Lysosphingolipid-induced calcium mobilization by H218 and Edg3 heterologously expressed in *Xenopus* oocytes. A: Increases in $^{45}Ca^{2+}$ efflux from *Xenopus* oocytes in response to various sphingolipids all at 300 nM final concentration. B: Concentration dependence of response to S1P. Data (mean ± S.E.M., $n=3$) are expressed as fold increase over basal. These experiments were replicated at least twice.

Taken together with the results of the SRE reporter gene assay, these data strongly suggest that H218 and Edg3 are functional receptors for S1P and perhaps other related lysosphingolipids.

SP and dh-SP did not evoke increases in $^{45}Ca^{2+}$ efflux from *Xenopus* oocytes, whereas they did activate SRE reporter gene in Jurkat cells. A possible explanation for this difference is the length of incubation times with lysosphingolipids in the two assays. The incubation time was 10 h in the SRE reporter gene assay and only 10 min in the $^{45}Ca^{2+}$ efflux assay. It is likely that SP and dh-SP were converted to the active ligands S1P and dh-S1P by sphingosine kinases from Jurkat cells over 10 h, but not in 10 min by *Xenopus* oocytes. These results of the two recombinant receptors are consistent with the previous demonstration that mitogenic effects of SP in fibroblasts required its conversion to S1P [1].

SPC has been reported to desensitize cells to S1P and vice versa, suggesting that SPC may share a common receptor with S1P [14]. We observed that SPC elicited signals through both H218 and Edg3 receptors in the SRE reporter gene assay with EC_{50} one order of magnitude higher than S1P. Thus, these related bioactive lysosphingolipids can indeed act at the same receptors with different potency.

The ligand for Edg1, the third orphan receptor in the group tested, remains unknown. It is possible that Edg1 is indeed a receptor for one of the lipid ligands tested but couples to signaling pathways not assayed in this study [30]. Alternatively, it is possible that Edg1 mediates responses to a distinct ligand.

S1P and SPC can elicit a variety of intracellular signaling events including mobilization of intracellular Ca^{2+}, and activation of pathways involving G_i-Ras-Raf-ERK and Rho GTPases [1,3,8,15]. The present study suggests that H218 and Edg3 can mediate at least two signaling responses: Ca^{2+} mobilization, and SRE-driven transcription which requires activation of both G_i-Ras-Raf-ERK and Rho GTPases [27]. Whether these and other signaling responses to lysosphingolipids are mediated by a single or multiple receptors in a given cell type is unknown. More importantly, the in vivo physiological and pathological functions of lysosphingolipid signaling remain to be explored. Identification of H218 and Edg3 as lysosphingolipid receptors provides new tools for defining the mechanisms of lysosphingolipid signaling and their physiological functions.

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