Characterization of a Novel Subtype of Human G Protein-coupled Receptor for Lysophosphatidic Acid*

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The recent identification of the Vzg-1/Edg2 protein as a functional G protein-coupled receptor for lysophosphatidic acid (LPA) has allowed a sequence-based search for new genes that may encode novel subtypes of LPA receptors. A human cDNA encoding a G protein-coupled receptor, designated Edg4, was identified by searching the GenBank® for homologs of the human Edg2 LPA receptor. The Edg4 protein is 46% identical and 72% similar in amino acid sequence to human Edg2. When overexpressed in Jurkat T cells, the Edg4 protein mediated LPA-induced activation of a serum response element reporter gene with LPA concentration dependence (EC₅₀ of 10 nM) and specificity. This LPA-induced reporter gene activation could be partially inhibited by pretreatment with pertussis toxin or C3 exoenzyme, suggesting requirements for both a G protein and Rho GTPase. Overexpression of Edg4 in Jurkat cells also led to increases in specific binding sites for [³H]LPA. Northern blots revealed that two edg4 mRNA transcripts of 1.8 and 8 kilobases are distributed very differently from edg2 mRNAs in adult human tissues and several cancer cell lines. The existence and distinctive tissue expression of structurally different subtypes of LPA receptors may provide one basis for tissue-specific functions and permit independent regulation of each subtype of LPA receptor.

Lysophosphatidic acid (LPA) is a lipid mediator with diverse biological activities (1, 2). LPA is generated by phospholipase cleavage of membrane phospholipids from stimulated cells, especially activated platelets (3). LPA is present at micromolar concentrations in serum and accounts for much of the cellular proliferative effect of serum (4–6). LPA elicits numerous biological functions in addition to proliferation, such as platelet aggregation, smooth muscle contraction, inhibition of neuroblastoma cell differentiation, chemotaxis, tumor cell invasion, and antiproliferative effects on some cell types (1, 2). The intracellular biochemical signaling events that mediate the effects of LPA include stimulation of phospholipase C and increases in cytoplasmic calcium concentration, inhibition of adenyl cyclase, and activation of phosphatidylinositol 3-kinase, the Ras-Raf-MAP kinase cascade, and Rho GTPases and Rho-dependent kinases (1, 2). The Ras-Raf-MAP kinase and Rho pathways stimulate the transcription factors ternary complex factor and serum response factor, respectively. Ternary complex factors and serum response factors synergistically activate transcription of immediate-early genes, such as c-fos, by binding to serum response element (SRE) in the promoters (7).

It has been demonstrated that specific G protein-coupled receptors (GPCRs) present on many types of cells mediate the cellular activities of LPA (2, 8, 9). Several groups have just reported the isolation of cDNAs encoding two structurally different GPCRs for LPA. Hecht et al. isolated a mouse cDNA termed ventricular zone gene-1 (vzg-1) encoding one such GPCR. When overexpressed in neocortical cells, the vzg-1 receptor mediated LPA-induced morphological changes, inhibition of adenyl cyclase, and increases in [³H]LPA-binding sites (10). Our laboratory identified human Edg2, a human homolog of vzg-1, as a functional receptor for LPA (11). Expression of Edg2 in HEK293 and Chinese hamster ovary cells led to elevated responses of LPA-induced SRE transcriptional reporter gene activation and increases in [³H]LPA-binding sites. The amino acid sequences of mouse vzg-1 and human Edg2 proteins are 96% identical, suggesting that they are the same LPA receptor in two different species. Guo et al. isolated a cDNA encoding a different GPCR from Xenopus oocytes (12). Overexpression of this cDNA in oocytes potentiated oscillatory Cl⁻ currents in response to LPA that could be attenuated by specific antiensiene oligonucleotides. This Xenopus LPA receptor shows little overall sequence similarity to the Vzg-1/Edg2 LPA receptors.

Some functional studies have implied that multiple subtypes of LPA receptors with distinctive signaling properties mediate diverse cellular effects of LPA (13, 14). To fully define the biological functions of LPA, we sought to identify novel subtypes of LPA receptors at a molecular level. In this study, we discovered a human cDNA termed edg4 encoding a GPCR with an amino acid sequence similar but not identical to human Edg2. We demonstrated that Edg4 is a novel subtype of LPA receptor functionally analogous to Edg2, but with a different tissue distribution.

EXPERIMENTAL PROCEDURES

Materials—Oleoyl-LPA, lysophosphatidyl-choline, lysophosphatidyl-ethanolamine, lysophosphatidyl-serine, phosphatidic acid (PA), sphingosine-1-phosphate, fatty acid-free human serum albumin, and bovine serum albumin were purchased from Sigma. The mammalian expression vector pCDEF3 (15), with a human elongation factor 1α promoter, was a generous gift from Dr. Jerome Langer (University of Medicine and Dentistry-New Jersey). Lipofectin and DMRIE-C lipofection reagents for transfection and OPTI-MEM medium were from Life Technologies, Inc. Jurkat leukemic T cells were obtained from Dr. Arthur
Weiss (UCSF). Cell culture media and fetal bovine serum were from UCSF Cell Culture Facilities. Pertussis toxin (PTX) was purchased from CalBiochem (La Jolla, CA). Recombinant Clostridium botulinum C3 ADP-ribose transferase (C3 exoenzyme), which specifically ADP-ribosylates Rho (16), was kindly provided by Drs. S. Narumiya and Y. Saito of Kyoto University (Kyoto, Japan). Human multiple tissue Northern blots were obtained from CLONTECH (Palo Alto, CA). Plasmid pGL3-basic and luciferase assay reagents were from Promega (Madison, WI). 1-Oleoyl [oleoyl-9,10-3H]LPA with a specific activity of 56.2 Ci/mmol was from NEN Life Science Products.

Cloning and Plasmid Constructs—The BLASTN program was used to search dbEST division of GenBank™ for sequence homologous to that of human edg2 (11). A DNA clone (GenBank™ accession number 755526) with its 5’ sequence (GenBank™ accession number AA419064) similar but not identical to the 5’ region of edg2 was identified and obtained from I.M.A.G.E. Consortium through Genome Systems (St. Louis, MO). The entire 1.7-kb insert of clone 755526 was sequenced on both strands using an ABI automated DNA sequencer (Howard Hughes Medical Institute DNA core facility, UCSF). The nucleotide sequence of 755526 was found to be highly homologous to that of human edg2 and therefore was redesignated human edg4. The 1.7-kb insert was cut out by EcoRI and NotI and subcloned into the mammalian expression vector pCD6F3. The 1.1-kb cDNA of human edg2 coding region was cleaved from Edg2/RSV (11) and also subcloned into pCD6F3. The 1.1-kb insert of Edg2/EF3 were labeled with 32P and used as probes in Southern blots. The 1.1-kb cDNA clone (GenBank™ accession number 755526) was sequenced and located on chromosome 19p12 (GenBank™ accession number AC002306). A cDNA clone (GenBank™ accession number 975526) with its 5’ region. The corresponding 1.7-kb cDNA clone (GenBank™ accession number 755526) contains the sequence of the full-length coding region of a novel protein that is 46% identical and 72% similar to the human Edg2 LPA receptor (Fig. 1). We designated this cDNA as human edg4 and its protein product as human Edg4 protein. The Edg4 protein consists of 382 amino acids with an estimated molecular weight of 42,626. It has some of the common features of a GPCR, including seven hydrophobic segments, potential N-linked glycosylation sites at the N terminus, and phosphorylation sites for serine/threonine kinases in the intracellular regions. Edg4 also possesses unique characteristics distinct from most other GPCRs. In Edg4, an alanine replaces a proline disulfide bond with another cysteine in the second extracellular loop in most other GPCRs.

Edg4 receptor gene plasmid 1.1-kb insert of Edg2/EF3 were labeled with 32P and used as probes in Southern blot analyses. Jurkat cells were chosen because they lack background responses to LPA in the SRE reporter gene assay. Given the high degree of sequence homology of Edg4 protein with previously characterized LPA receptors Edg2 and Vzg-1, we predicted that Edg4 was a receptor for LPA or a structurally similar lipid modulator. Edg4 protein was therefore expressed in Jurkat T cells for studies of LPA-induced responses and of radioactive LPA binding. Jurkat cells were chosen because they lack background responses to LPA in the SRE reporter gene assay.

When co-transfected with the SRE-luciferase reporter gene, Edg4, as Edg2, mediated increases in SRE-driven reporter gene expression induced by 1 μM LPA and, to lesser extent, PA.
(Fig. 2). The structurally related lipids lysophosphatidyl-choline, lysophosphatidyl-ethanolamine, lysophosphatidyl-serine, and sphingosine 1-phosphate at a concentration of 1 μM failed to generate significant increases in luciferase expression (Fig. 2). The control Jurkat cells transfected with empty pCDEF3 vector showed minimal changes in response to LPA or other phospholipids (Fig. 2).

The activation of LPA-induced SRE-driven reporter gene in Edg4- and Edg2-transfected Jurkat cells was significant at 1 nM LPA, reached a maximum at 100 nM LPA, and exhibited an EC50 of approximately 10 nM (Fig. 3A). PA mimics the effect of LPA in both Edg4- and Edg2-transfected Jurkat cells but with much higher EC50 values of at least 500 nM (Fig. 3B). The magnitude of LPA- and PA-evoked SRE-driven reporter activation of Edg4 was approximately four times higher than that of Edg2 (Figs. 2 and 3).

Pertussis bacterial toxin and botulinum C3 exoenzyme specifically inactivates Gα and Rho, respectively. LPA-induced activation of the SRE reporter gene in both Edg2- and Edg4-transfected Jurkat cells was partially blocked by PTX or C3 exoenzyme pretreatment (Fig. 4). The two toxins added together further inhibited the effects of LPA. These results suggest that both α subunit of Gi and Rho GTPase are involved in transducing signals from the Edg2 and Edg4 receptors to the SRE reporter gene activation.

[3H]LPA binding is generally characterized by a substantial nonspecific component due to its amphiphilic binding to cell membranes. Using intact Jurkat T cells, we were able to reduce nonspecific binding of [3H]LPA compared with that of membrane preparations. The background-specific [3H]LPA binding in the control vector-transfected Jurkat cells was 1409 ± 123 cpm (mean ± S.E., n = 3). In the same number of Edg4-transfected Jurkat cells, the specific binding was 3082 ± 298 cpm (mean ± S.E., n = 3), which was significantly higher than the controls (p < 0.01). When calculated in terms of receptor density, control Jurkat cells had 15,000 ± 1300 LPA-binding sites and Edg4 transfectants had 33,000 ± 3200 binding sites. Thus, Edg4 overexpression in Jurkat cells resulted in increases in the number of specific binding sites for LPA. The other lysophospholipids (lysophosphatidyl-choline, lysophosphatidyl-ethanolamine, and lysophosphatidyl-serine) failed to competitively inhibit [3H]LPA binding to vector- or Edg4-transfected Jurkat cells (data not shown).

The distribution of mRNA encoding edg2 and edg4 in a spectrum of human tissues and cancer cells was examined by Northern blot analyses (Fig. 5). The edg2 transcripts were found in almost all human tissues with the highest abundance in brain and the lowest abundance in liver and peripheral blood leukocytes. The edg2 transcripts also were detected in HeLa carcinoma, SW480 colorectal adenocarcinoma, A549 lung carcinoma, and G361 melanoma but were undetectable in HL60
promyelocytic leukemia, K562 chronic myelogenous leukemia, MOLT-4 lymphoblastic leukemia, and Raji Burkitt’s lymphoma cells (Fig. 5).

In contrast, the two major edg4 transcripts of 8 and 1.8 kb were not represented in human tissues as widely as edg2 transcripts and showed a pattern of distribution completely different from edg2 (Fig. 5). The 8-kb transcript was detected in peripheral blood leukocytes, thymus, and spleen, whereas the 1.8-kb transcript was in the leukocytes, testis, prostate, and pancreas. The edg4 transcripts were almost undetectable in brain, heart, placenta, and digestive tract where edg2 transcripts were abundant but were found in leukocytes where edg2 was undetectable. In cancer cells, the 8-kb transcript of edg4 was found in all cell types, whereas the 1.8-kb transcript was only detected in HeLa, SW480, and A549 cells where edg2 transcripts were also more abundant. In addition, a minor transcript of 2.8 kb was seen in G361 and SW480 cells (Fig. 5).

**DISCUSSION**

The recent characterization of the Vzg-1/Edg2 proteins as functional LPA receptors has allowed a sequence-based search for new genes that may encode novel LPA receptors. A human cDNA, designated edg4, was identified by searching the dbEST and found to encode a GPCR that resembles the human Edg2 LPA receptor (Fig. 1). An SRE reporter gene assay demonstrated that Edg4 is a novel subtype of functional LPA receptor. Compared with Edg2, Edg4 was more active in stimulating SRE-driven transcription in transfected Jurkat T cells (Figs. 2 and 3). In several representative experiments, no major difference in the levels of Edg2 and Edg4 mRNAs was observed in the transfected cells by Northern blot analyses (data not shown). However, because the cell surface expression levels of Edg2 and Edg4 receptor proteins were not measured in the current study, the observed differences in activities could mean either that Edg4 protein was expressed at a higher level in these cells or that Edg4 had a higher intrinsic receptor activity than Edg2. That Edg4 is an LPA receptor was also substantiated by increases in binding sites of [3H]LPA to Edg4-transfected Jurkat cells. Due to extremely high nonspecific binding of amphiphilic LPA to cell membranes and the presence of background-specific binding in untransfected cells, reliable pharmacological binding parameters of Edg4 (e.g. $B_{max}$ and $K_d$) were not obtainable.

Untransfected and vector-transfected Jurkat cells displayed a significant number of specific binding sites to [3H]LPA and a weak LPA-induced calcium mobilization response (data not shown), whereas they did not respond to LPA in the SRE reporter gene assay (Figs. 2 and 3). This discrepancy is probably attributable to the presence of LPA receptors of a human version of the *Xenopus* LPA receptor (12) or an as yet unidentified subtype in Jurkat cells.

PA has been implicated in the activation of cellular kinases, phospholipases, and small GTPases in some cell types, including neutrophils (20, 21). Although many of the reported effects of PA can be attributed to its metabolites, PA is clearly a messenger of intracellular and extracellular signaling (20, 21). Our results demonstrated that PA-induced activation of SRE-driven reporter gene was dependent on Edg2 and Edg4 transfection. However, the EC$_{50}$ of PA was more than 100-fold high than that of LPA. This suggests that PA, if it acts directly on Edg2 and Edg4 receptors, is a much weaker agonist for these receptors.

**FIG. 4.** Inhibition of LPA-induced activation of SRE-driven luciferase expression by PTX and C3 exoenzyme. During serum starvation and LPA treatment, cells were incubated in the absence or the presence of 50 ng/ml of PTX or 10 $\mu$g/ml C3 exoenzyme or the two toxins together. The activation of the reporter gene in controls without toxins are 4.78 ± 0.22-fold for Edg2, and 15.6 ± 1.09-fold for Edg4. Data are plotted as percentages of respective controls (Ctrl) without toxins. Values are the means ± S.E. (n = 6) of a representative of three experiments.

**FIG. 5.** Northern blot analysis of edg2 and edg4 expression. 2 $\mu$g of poly(A)$^+$ RNA from indicated adult human tissues and cancer cells were hybridized with $^{32}$P-labeled probes of edg2 (upper panel) or edg4 (lower panel) under high stringency conditions. Molecular mass markers are indicated on the left in kb.
Very different human tissue distribution of edg4 and edg2 mRNAs was revealed by Northern blots analyses (Fig. 5). In adult human brain, heart, placenta, and digestive tissues where edg2 mRNAs are abundant, edg4 mRNAs are undetectable. In peripheral leukocytes where edg2 mRNAs are undetectable, edg4 mRNAs are the most abundant among all tissues. The specific type(s) of leukocytes containing edg4 mRNAs and the physiological functions mediated by Edg4 in these cells remain to be discovered.

The tissue-specific expression of edg2 and edg4 was further extended to eight different cultured cancer cell lines. Two major transcripts of edg4 also exhibit tissue- and cancer cell-specific expression profiles. The sequences and their translated protein products of the edg4 transcripts remain unknown. Nevertheless, it is noted that the cultured cancer cells that express edg2 and the 1.8-kb transcript of edg4 are adherent cells grown in culture, whereas those grown in suspension (HL60, K562, Molt, and Raji) do not express a significant level of edg2 or the 1.8-kb transcript of edg4. The correlation between the various edg2 and edg4 transcripts, their translated receptor proteins, and their functions in cell adhesion and contact deserves further investigation. Nonetheless, the gene expression of edg2 and edg4 is likely to be regulated differently in different tissues and cells.

LPA receptors in fibroblasts couple to at least three distinct G proteins, namely Gq, Gi, and G12/13 (2). Activation of Gq stimulates phospholipase C and a consequent mobilization of intracellular calcium. Activation of PTX-sensitive Gi inhibits adenyl cyclase and stimulates the Ras-Raf-ERK pathway leading to transcriptional activation mediated by ternary complex factors. Activation of the PTX-resistant G12/13 stimulates Rho, which leads to actin-based cytoskeleton changes and transcriptional activation mediated by serum response factor. The Gq- and Rho-activated pathways synergistically stimulate transcription of many growth-related genes containing SRE in their promoters (7). Previous studies of Hecht et al. demonstrated that mouse Edg2 (Vzg-1) mediated inhibition of adenyl cyclase via a PTX-sensitive Gi protein and cell morphological changes via PTX-insensitive, presumably Rho-related GTPases (10). Our results indicate that Edg2- and Edg4-mediated activation of SRE-driven transcription in Jurkat cells involves a PTX-sensitive Gi protein and C3 exoenzyme-sensitive Rho GTPase (Fig. 4). We were unable to determine whether Edg2 and Edg4 also couple to Gq, which would lead to an increase in formation of inositol phosphates and intracellular calcium mobilization, because there were considerable background calcium responses to LPA in all untransfected cells tested.

In conclusion, we have discovered a human cDNA encoding a novel GPCR, named Edg4, which has sequence similar to the previously identified human Edg2 LPA receptor. Using an SRE-driven reporter gene assay and a [3H]LPA binding assay, we have demonstrated that Edg4 is a novel functional receptor for LPA. Northern blot analysis revealed that mRNAs for edg2 and edg4 have strikingly different tissue and cell distribution. The existence and distinctive tissue expression of structurally different LPA receptors may provide one basis for tissue-specific functions of LPA and permit independent regulation of each subtype of LPA receptor.

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