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Research Article

Differential stimulation of signaling pathways initiated by Edg-2 in response to lysophosphatidic acid or sphingosine-1-phosphate

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Abstract. Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are produced during cell activation and have multiple effects on cells. A family of seven transmembrane-spanning domain G-protein-coupled receptors, named Edg, mediate these effects of LPA and S1P. In this study, transient overexpression of Edg-2 sensitized MG63 human osteosarcoma cells to both LPA- and S1P-mediated stimulation of fibronectin matrix deposition and actin stress fiber formation. Both lipids were active in the 1–20 nM concentration range on cells transfected with Edg-2 as compared to the 10–200 nM range on mock-transfected cells. The signaling pathway for matrix deposition by Edg-2-transfected cells was Rho dependent. Overexpression of Edg-2 also caused a tenfold decrease in the concentration of either LPA or S1P that activated MAPKinase (Erk1/2) in MG63 cells. LPA- or S1P-stimulated activation of Erkl/2 was Gi dependent. These results indicate that, in MG63 cells, Edg-2 mediates actin stress fiber formation, fibronectin matrix assembly, and MAPKinase activation in response to either LPA or S1P.

Key words. MG63 cells; Edg-2; LPA; S1P; fibronectin; MAPKinase; GFP; Rho.

Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are produced during cell activation [1–7] and have multiple effects on cells, including actin stress fiber formation, up-regulation of cell surface fibronectin (FN) matrix assembly sites, and activation of the MAP-Kinase cascade [7–10].

Failure of intracellularly microinjected LPA to mimic the response of extracellular LPA indicated the presence of a cell surface receptor for LPA [3, 11]. S1P acts as an intracellular second messenger but also through cell surface receptors [12]. Recently, a family of seven transmembrane spanning G-protein coupled receptors for LPA and S1P has been described, namely Edg receptors (due to sequence homology to the endothelial differentiation gene, edg-1 [13]). Based on gene structure and protein homology, these receptors can be grouped into two subfamilies: the receptors for LPA consisting of Edg-2, Edg-4, and Edg-7, and the receptors for S1P consisting of Edg-1, Edg-3, and Edg-5 [7, 10, 14–22].

Edg-1, in addition to being a receptor for S1P, has been reported to be a low affinity receptor for LPA [23]. The structural similarity between LPA and S1P, the similarity in their effects, and cross-desensitization experiments [24, 25] raise the possibility that LPA and S1P may share, in some fashion, all of the Edg receptors. To

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address this question, we tested the effects of Edg-2 overexpression in MG63 cells using three endpoints: actin stress fiber formation, FN matrix assembly, and Erk1/2 activation. We found evidence that, in addition to being a receptor for LPA, Edg-2 is either a receptor or sensitizes other receptors for S1P in MG63 cells.

Materials and methods

Materials. The following were purchased: 1-oleoyl-LPA (Avanti Polar Lipids, Birmingham, Ala.); rhodamine-labeled phalloidin and fluorescein isothiocyanate (FITC; Molecular Probes, Eugene, Ore.); *Pertussis* toxin (PTX; List Biological Laboratories, Campbell, Calif.); S1P (LC Laboratories, Woburn, Mass.); bovine serum albumin (BSA; fraction V or fatty acid free), sphingosylphosphorylcholine, sphingomyelin, and phosphatidic acid (Sigma, St. Louis, Mo.); and polyclonal Anti-Active MAPK antibodies (Promega, Madison, Wisc.). Recombinant *Botulinum* C3 exotransferase was a generous gift from Dr. Connie Lebakken at the University of Wisconisin (Madison, Wisc.).

Cell transfection. MG63 osteosarcoma cells were obained from the American Type Culture Collection (Rockville, Md.). Cells were cultured in DMEM (Life, Technologies, Gaithersburg, Md.) supplemented with 5% fetal bovine serum (Intergen, Purchase, N.Y). For most studies, cells were seeded in 24-well cluster tissue culture plates (Costar, Cambridge, Mass.) the day before analysis.

Prior to transfection experiments, subconfluent cells were resuspended at 5×10^6 cells/ml in DMEM containing 10% fetal bovine serum. A total of 8 μg of plasmid was mixed with 400 μl of cell suspension and placed in a 0.4-cm electroporation cuvette (Bio-Rad, Hercules, Calif.). For cotransfection experiments, 4 μg of each plasmid was used. Transfection was accomplished at 280 V and 960 μF on the Gene Pulser with capacitance extender (Bio-Rad). The cell suspension was then placed in a 35-mm diameter tissue culture plate containing 2 ml of complete medium. Fresh complete medium was applied to the cells 1–2 h after electroporation. Cells were analyzed 72 h after transfection. Flow cytometry on a Becton Dickinson FACScan (Becton Dickinson, Mountain View, Calif.) of cells transfected with a plasmid (pEGFP-C1, generous gift of Dr. F. Bourre, UMR 5533 CNRS, France) coding for the green fluorescent protein (GFP) indicated that the transfection efficacy was consistently between 45% and 50% of the total cell population. To average out variations in transfection efficiency, at least three independent transfections using the same pool of cells were carried out in parallel for a given plasmid. Two days after transfection and 1 day before assay, cells transfected with the same plasmid were trypsinized, mixed together, and cultured at the appropriate density.

PTX and C3 exotransferase treatment were begun 48 h after transfection. Cells were seeded at a density of 1.5×10^5 cells/well and incubated for 24 h in complete medium containing 100 ng/ml of PTX or 25 μg/ml of C3 exotransferase before analysis of MAPKinase activation or FITC-FN binding (as described below).

Molecular biological techniques. Cloning human Edg-2 cDNA was carried out by heterologous RT-PCR from total RNA isolated from MG63 cells using the RNAgents Total RNA Isolation System (Promega). RNA (10 ng) and specific primers (1 μM) were subjected to one-tube reaction of reverse transcription and amplification by PCR using the Access RT-PCR system (Promega) following the manufacturer's instructions. PCR primers were based on the mouse Edg-2 (Vzg-1) cDNA sequence (GenBank accession number U70622) and a human sequence identified in the dbEST database. The primer sequences were: mouse-Vzg-1, nucleotides 218–235, 5′CAGCACTGTCATGGAGC3′; mouse-Vzg-1, nucleotides 1333–1314, 5′CTGGCTTCCTTCTAAACCAC3′; dbEST, nucleotides 53–72, 5′AATCGAGAGGCACATTACGG3′; dbEST, nucleotides 481–462, 5′TGTGGACAGCACACGTCTAG3′. Two overlapping PCR fragments, containing the 5′ and the 3′ sequences of human Edg-2, were T/A cloned into the mammalian expression pTargeT, and three clones sequenced in each case using the AmpliTaq FS Dye Terminator Ready Reaction Kit (Perkin-Elmer, Norwalk, Conn.). Using the unique *Xba*I restriction site present in each cDNA fragment, the full-length human cDNA was inserted into the pTargeT vector to yield pEdg-2.

Expression of human Edg-1, -2, -3, or -4 mRNA was analyzed by RT-PCR from total RNA isolated from MG63 cells as described by Zhang et al. [26]. Edg-5 was analyzed using the following primers: nucleotides 412–431, 5′AAGCTGTATGGCAGCGACAA3′, nucleotides 625–644, 5′ACGCAGTAGTGCGCACGTA3′.

Fluorescence microscopy. Isolation of plasma FN, labeling with FITC, and FITC-FN deposition were carried out as described previously [26–28]. Briefly, cells cultured on coverslips were incubated with 20 μg/ml FITC-labeled FN in the absence or presence of various additives for 1 h at 37 °C, and fixed in 3% paraformaldehyde for 30 min. For actin localization, fixed cells were permeabilized with 0.2% Triton X-100 for 5 min, and incubated with 0.1 μg/ml rhodamine-labeled phalloidin for 20 min. Cells were viewed on a BX-60 fluorescent microscope (Olympus America, Melville, N.Y.).

Stimulation of actin stress fiber formation was quantified after cell cotransfection with pEGFP-C1 and pTargeT or pEdg-2 as described above. Each lipid concentration was tested in triplicate, and for each repli-

cate, 50 cells expressing GFP were scored for the presence or absence of actin filaments.

Detection of activated MAPKinase. Cells were starved for 4 h in serum-free DMEM containing 0.2% fatty-acid-free BSA and then incubated in the absence or presence of various additives for 5 min at 37 °C. The cells were washed twice with prechilled TBS at 4 °C, placed on ice, and solubilized in SDS sample buffer (2% SDS, 10% glycerol, and 50 mM Tris-HCl, pH 6.8) followed by heating at 100 °C for 10 min. An aliquot was saved for protein quantification using the BCA protein assay reagent (Pierce, Rockford, Ill.), and the remaining cell lysate was adjusted to 300 mM β-mercaptoethanol and 0.0025% bromophenol blue and heated again for 5 min. Equivalent amounts of protein from each sample (10 μg) were separated by SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore, Bedford, Mass.). The double phosphorylated forms of MAPKinase (Erk1 and Erk2) were detected by probing the membranes with Anti-Active MAPK polyclonal antibodies according to the manufacturer's instructions. The immunoreactivity was detected by a peroxidase-labeled anti-rabbit secondary antibody, and immunoreactive bands were visualized by chemiluminescence (NEN Life Science Products, Boston, Mass.). Autoluminoradiograph films were scanned, and the relative amount of double-phosphorylated forms of Erk1/2 was quantified from densitometry using NIH Image software. For each lipid stimulation, the specific increase of the Erk1/2 phosphorylated form was calculated using the following equation:

$$\frac{(D_x - D_0)}{(D_E - D_0)} \times 100$$

where D_0 corresponds to phosphorylated Erk1/2 detected in the absence of lipid, D_E corresponds to phosphorylated Erk1/2 detected at a dose of LPA (10 nM) or S1P (10 μM) for which phosphorylation was equivalent in mock-transfected and pEdg-2-transfected cells, and D_x corresponds to phosphorylated Erk1/2 detected at a specific concentration (X) of lipid.

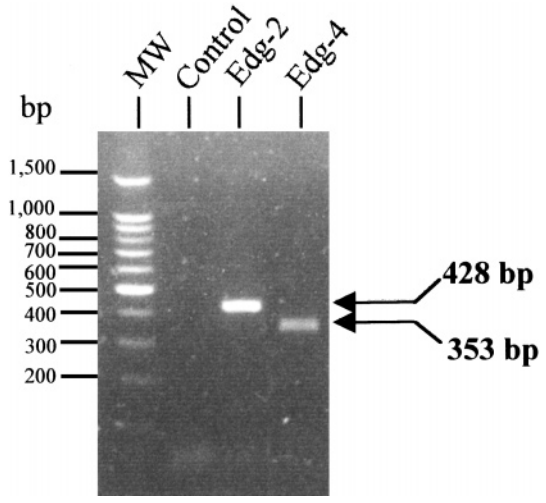

Figure 1. Expression of LPA receptor mRNAs in human MG63 osteosarcoma cells. RT-PCR analysis of Edg-2 and Edg-4 expression was carried out with MG63 cells RNA. The negative control experiment (Control) was performed in the absence of the avian myeloblastosis virus reverse transcriptase. The specific amplification products for Edg-2 and Edg-4 transcripts are 428 and 353 bp, respectively. MW, molecular weight markers; bp, base pairs.

Results

Overexpression of Edg-2 sensitizes MG63 cells to LPA- and S1P-stimulated deposition of FITC-FN in the Rho-dependent signaling pathway. Heterolgous RT-PCR allowed us to detect mRNA expression for Edg-2 and Edg-4 LPA receptors in MG63 osteosarcoma cells (fig. 1), and clone the full-length Edg-2 cDNA sequence from MG63 cell RNA. This sequence exhibited nearly complete identity to Edg-2 cloned from a human lung cDNA library [29]. Our sequence encoded a Gly rather than a Ser at codon 340. Beside mRNA expression for LPA receptors, Edg-5 was the only S1P receptor detected by RT-PCR in MG63 cells [26; D. Mosher, unpublished results].

Transfected MG63 cells were tested for their ability to bind FITC-FN upon LPA or S1P stimulation as assessed by fluorescence microscopy (fig. 2A). Cells transfected with empty pTargeT vector or transfected with pEdg-2 exhibited a similar low background of FITC-FN deposition in the absence of stimulation and a similar high intensity of fibrillar fluorescence with 500 nM LPA or 200 nM S1P, indicative of increased deposition of FITC-FN. The fibrillar fluorescence, when present, extended between and over most of the cells. Cells transfected with pEdg-2 exhibited increased deposition of FITC-FN induced by 30 nM LPA compared to cells transfected with pTargeT (fig. 2A). This increased FITC-FN deposition by pEdg-2-transfected cells stimulated with 30 nM of LPA was abolished upon pretreatment with *Botulinum* C3 exotransferase (a specific inhibitor of the small G protein Rho; fig. 2B), indicating that the FN matrix assembly site on pEgd-2-transfected MG63 cells was mediated by a signal transduction pathway dependent on Rho activation. Cells transfected with pEdg-2 also exhibited enhanced deposition of FITC-FN induced by S1P stimulation at a dose (20 nM) that was still ineffective with cells transfected with pTargeT (fig. 2A). In all experiments, a tenfold higher concentration of LPA or S1P was necessary to observe the same intensity of fluorescence in cells trans-

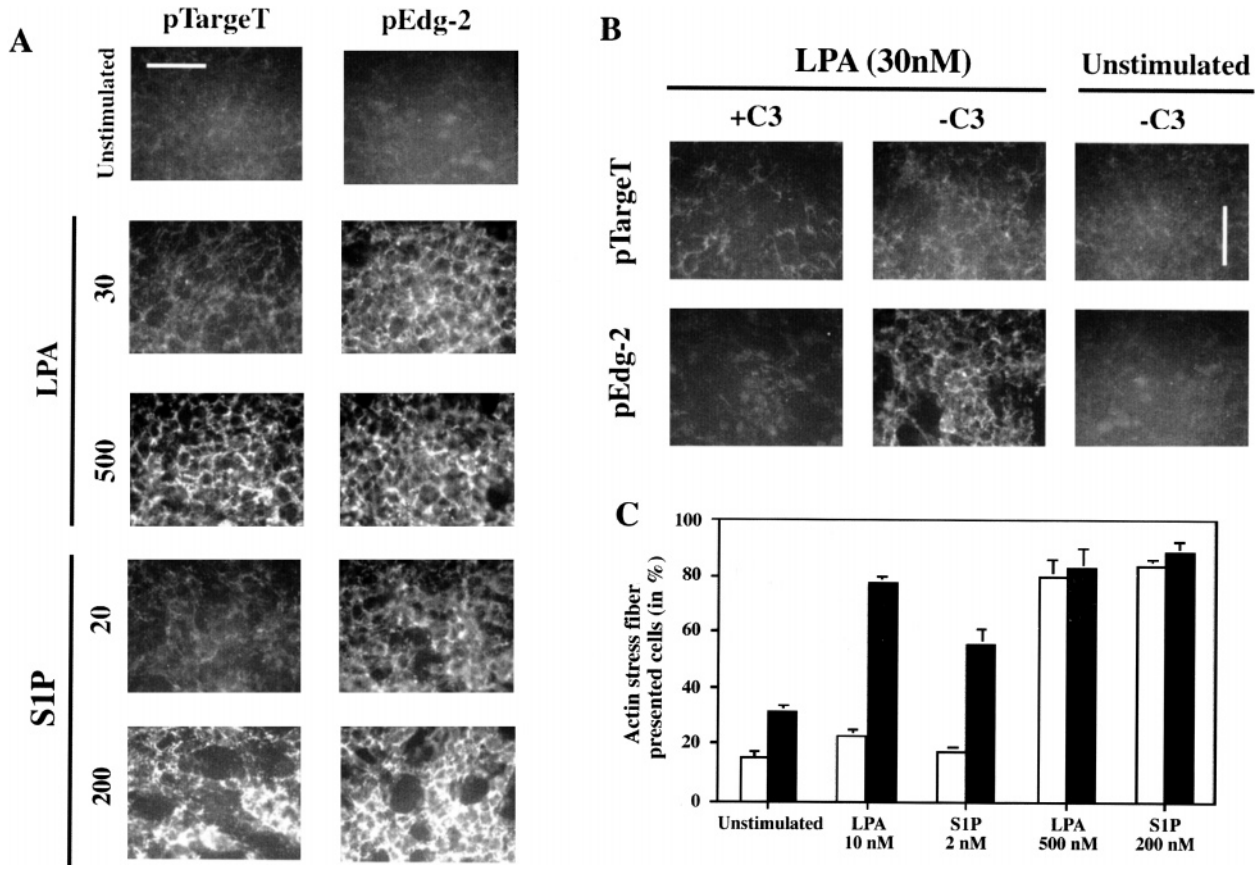

Figure 2. FITC-FN binding and actin stress fiber formation in mock (pTargeT)- and Edg-2 (pEdg-2)-transfected MG63 cells stimulated by LPA or S1P. (*A*) Cells were incubated at 37 °C for 1 h with FITC-labeled FN (20 μg/ml) in the absence (unstimulated) or presence of LPA or S1P (doses indicated in nM). Photographs are from a single experiment and are representative of three independent experiments. (*B*) Effect of C3 exotransferase treatment on LPA-stimulated FITC-FN binding on pTargeT- and pEdg-2-transfected cells. Cells were preincubated for 24 h at 37 °C in complete medium with (+ C3) or without (− C3) 25 μg/ml of C3 exotransferase. FITC-FN binding was then carried out in the absence (unstimulated) or presence of LPA (30 nM). Phototgraphs are from a single experiment and are representative of two independent experiments. Bar, 110 μm. (*C*) Actin stress fiber formation in transfected cells stimulated by LPA or S1P. MG63 cells were cotransfected with a GFP-coding plasmid pEGFP-C1 and pTargeT (open bars) or pEdg-2 (black bars). Cells were incubated at 37 °C for 1 h in the absence (unstimulated) or presence of LPA or S1P at the indicated concentrations. Cells were washed, fixed, permeabilized, and incubated with rhodamine-conjugated phalloidin. GFP-expressing cells were scored under fluorescence microscopy for the presence of actin stress fibers. Data are expressed as the mean (± SEM) percentage of stress-fiber-presenting cells (triplicate determination) and are representative of two independent experiments.

fected with pTargeT (concentration range 10–200 nM) compared to cells transfected with pEdg-2 (concentration range 1–20 nM) (results not shown).

LPA and S1P induced stress fiber formation in Edg-2 MG63 transfected cells. Because LPA stimulated FN matrix assembly concomitant with cell shape changes, actin stress fiber formation, and cell contraction [28], we used these three parameters as endpoints to analyze individual MG63 cells cotransfected with GFP-coding plasmid (pEGFP-C1) and either pTargeT or pEdg-2. Cells were stained with rhodamine-conjugated phalloidin, and scored for the expression of GFP and presence of actin stress fibers (fig. 2C). Of MG63 cells cotransfected with pEGFP-C1 and pEdg-2, 77% or 55% had enhanced actin stress fibers when stimulated with 10 nM LPA or 2 nM S1P, respectively. In contrast, only 23% or 15% of MG63 cells cotransfected with pEGFP-C1 and pTargeT had enhanced stress fibers when similarly stimulated by LPA or S1P. The same high percentages of GFP-expressing cells with actin stress fibers (80–88%) were observed with Edg-2 or mock-transfected cells were incubated with 500 nM LPA or 200 nM S1P.

LPA and S1P induced activation of MAPKinase in Edg-2-transfected cells in a G_i-dependent signaling pathway. LPA and S1P are known to induce the activation

of the MAPKinase cascade [8, 9]. Doubly phosphorylated Erk1 and Erk2 representing the activated forms of these proteins [30] were detected in MG63 cell lysate by Western blotting using anti-active MAPKinase polyclonal antibodies. In the absence of stimulation, basal activation of Erk1/2 was detected in MG63 cells trans-

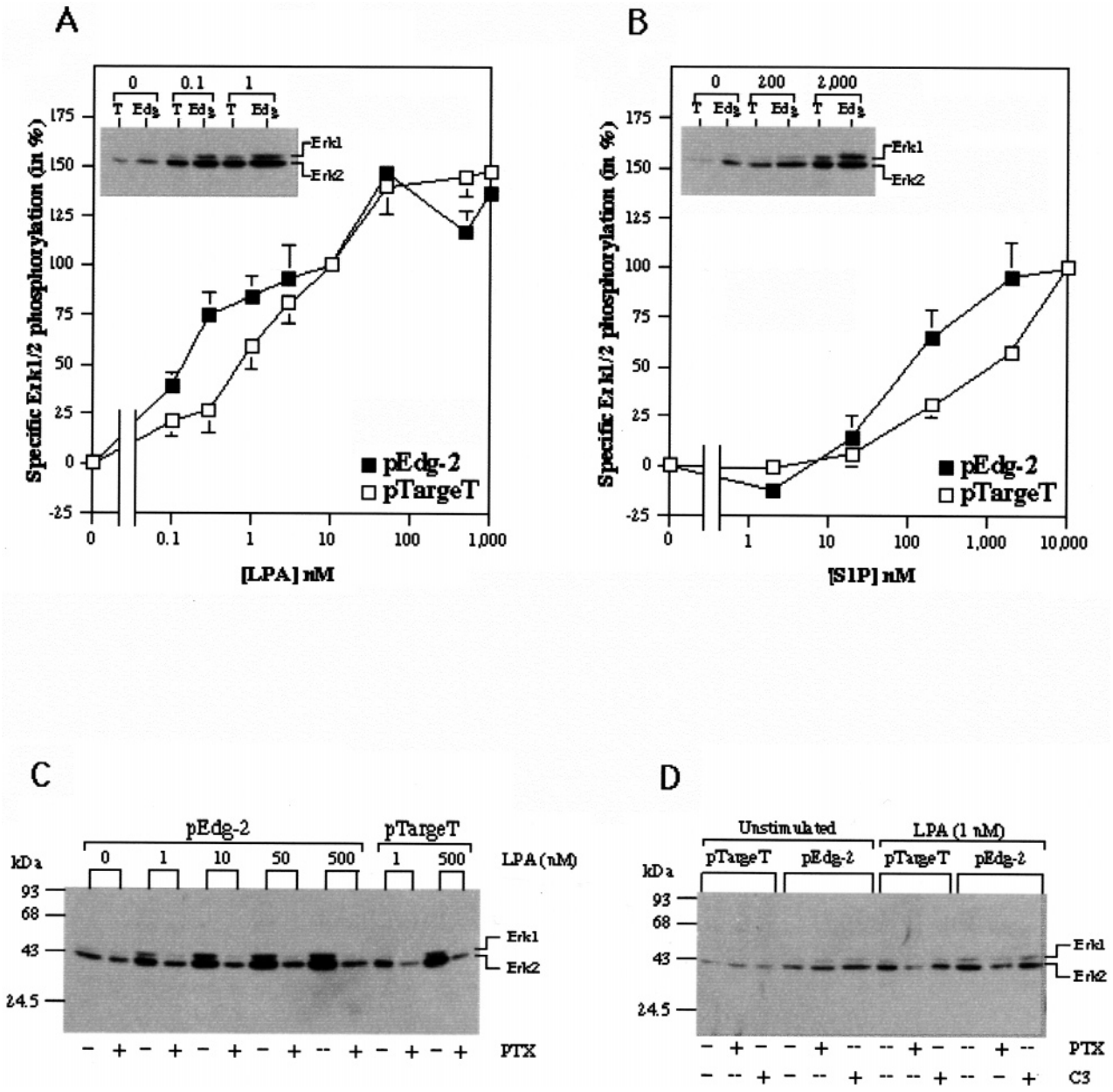

Figure 3. Dose-response curves of LPA- and S1P-mediated phosphorylation of Erk1/2 in mock (pTargeT)- and Edg-2 (pEdg-2)-transfected MG63 cells. Serum-deprived cells were exposed for 5 min to increasing concentrations of LPA or S1P (nM). After stimulation, cells were lysed and 10 μg of protein/lane was resolved on SDS-PAGE and then immunoblotted to detect the presence of the double phosphorylated form of MAPKinase, Erk1 and Erk2. (*A*, *B*) Western blots were scanned and the densitometry of Erk1/2 band intensity is reported as the specific increase of the Erk1/2 phosphorylated form (%) relative to the concentration of lipid that caused equal phosphorylation of pTargeT- and pEdg-2-transfected cells. Insets, representative Western blots for mock (T)- and Edg-2-transfected cells stimulated with LPA (0.1 and 1 nM) or S1P (200 and 2000 nM). Data represent means ± SEM from five (*A*) and four (*B*) independent experiments. (*C*, *D*) LPA-mediated phosphorylation of Erk1/2 in MG63 cells transfected with pTargeT or pEdg-2 was monitored after pretreatment with PTX (*C*) or C3 exotransferase (*D*). Transfected cells were incubated with (+) or without (−) either PTX (100 ng/ml) or C3 (25 μg/ml) for 24 h at 37 °C in complete medium, serum deprived for 4 h and then exposed to various concentrations of LPA (nM) for 5 min or left unstimulated. Data are representative of three (*C*) and two (*D*) independent experiments.

fected with either pTargeT or pEdg-2 (fig. 3A, B, insets). Densitometry of Western blots revealed that pEdg-2-transfected cells had 3.1 ($\pm$0.4)-fold (mean $\pm$ SEM, n = 10) higher endogenous activation of Erk1/2 than mock-transfected cells (fig. 3A, B). LPA and S1P activated Erk1/2 in mock-transfected cells with an EC_{50} of approximately 1 nM and 1000 nM, respectively (figs. 3A, B). In contrast, pEdg-2-transfected cells exhibited a tenfold decrease in the concentration required for Erk1/2 activation by LPA ($EC_{50} \sim$ 0.1 nM) and S1P ($EC_{50} \sim$ 100 nM) (figs. 3A, B).

Pertussis toxin (PTX), a specific inhibitor of G_i proteins, completely inhibited Erk1/2 activation induced by an increasing concentration of LPA (fig. 3C) or S1P (results not shown). However, basal activation of Erk1/2 in both mock- and Edg-2-transfected cells was not altered by PTX preincubation (fig. 3D).

C3 exotransferase inhibits integrin-dependent Erk activation in NIH 3T3 cells, indicating the potential participation of Rho in pathways leading to Ras and Erk activation [31, 32]. We compared effects of C3 exotransferase and PTX treatment on Erk1/2 activation in MG63 cells transfected with pEdg-2 (fig. 3D). In contrast to PTX pretreatment, C3 exotransferase pretreatment had no effect on LPA stimulation of Erk1/2 activation. Like PTX, C3 exotransferase did not block basal Erk1/2 activation.

Discussion

Edg-1, first identified as an S1P receptor has also been described to interact with LPA to induce MAPKinase activation and Rho-dependent cell shape change [23]. Overexpression of Vzg-1, the mouse counterpart of human Edg-2, in COS cells or *Xenopus* oocytes as well as Edg-2 overexpression in SF9 insect cells did not potentiate LPA-induced Ca^{2+} mobilization or MAPKinase activation [21, 33]. Because LPA can clearly stimulate cells through Edg-2 [19, 20, 34], the lack of effect of LPA on Edg-2-transfected COS and SF9 cells [21, 33] suggests that appropriate intracellular signaling molecules (i.e., G-proteins) must be present to transduce signals upon binding of LPA to Edg-2. Indeed, the absence of S1P stimulation of *Xenopus* oocytes was overcome by coexpression of one of the S1P receptors (Edg-1, Edg-3, or Edg-5) and individual subtypes of G-proteins [35]. Thus, overexpression of Edg-2 in known LPA-responsive cells that constitutively express Edg-2 may circumvent the problem of not having appropriate intracellular signaling molecules. In the present study, we transiently overexpressed Edg-2 in Edg-2-expressing cells (human MG63 osteosarcoma cells) and found that Edg-2-transfected cells were sensitized to both LPA and S1P.

Both LPA and S1P are active in the 10–200 nM concentration range to stimulate FN matrix deposition by MG63 cells [26, 36]. Sensitivity of MG63 cells to S1P is not dependent on a process mediated by secondary release of endogenous LPA [26]. In the present study, a 1–20 nM range of concentration of either lipid was sufficient to stimulate FN matrix deposition by Edg-2-transfected MG63 cells whereas mock-transfected cells required a tenfold higher concentration. Sensitization of Edg-2-transfected MG63 cells to enhanced FN deposition was dependent on the small G-protein Rho-dependent signaling pathway and was associated with sensitization to enhanced actin stress fiber formation. These findings are in agreement with the observation that Edg-2 activates a Rho-dependent SRE-driven reporter gene [29].

Overexpression of Edg-2 in MG63 cells caused a tenfold decrease in the concentration of either LPA or S1P that activated Erk1/2. Both LPA- and S1P-stimulated activation of Erk1/2 were completely lost in the presence of PTX but not after C3 treatment, indicating that the signaling pathway for Erk1/2 activation was G_i-dependent and Rho independent. These findings are in agreement with the fact that LPA and S1P activate MAPKinase in Rat-1 or Swiss 3T3 fibroblasts via a PTX-sensitive Ras-dependent pathway [6, 37, 38]. Dose-response data for MG63 cells are in accordance with previous estimations of EC_{50}s for Erk1 activation by LPA ($\sim$40 nM) in Rat-1 cells [39] and S1P ($\sim$1 μM) in Swiss 3T3 fibroblasts [6].

Activation of Erk1/2 in mock-transfected MG63 cells required lower concentrations of LPA ($EC_{50} \sim$1 nM) than enhancement of FN assembly ($EC_{50} \sim$30 nM) whereas greater doses of S1P were required for activation of Erk1/2 ($EC_{50} \sim$1 μM) than for enhancement of FN assembly ($EC_{50} \sim$100 nM). Overexpression of Edg-2 in MG63 cells also caused a threefold increase in baseline activity of Erk1/2. Increased binding of ^{35}S-GTPγS to G-proteins has been reported in RH7777 hepatoma cells transfected with Edg-2 [20]. In addition, ligand-independent activation of signaling pathways that sensitize the cells to anoikis have been described in the ovarian cancer cell line A2780 transfected to overexpress Edg-2 [40]. Similar increases in baseline activity were noted upon overexpression of Edg-1 in human embryonic kidney 293 cells [41] and upon overexpression of the β_2-adrenergic receptor in transgenic mice [42]. The phenomenon has been explained by the 'two-state model' of G-protein coupled receptor activation [43], whereby the receptor is in equilibrium between the inactive conformation and a spontaneously active conformation that can couple to G-protein in the absence of ligand. According to this model, the active conformation of Edg-2 would be increased in Edg-2-transfected MG63 cells.

It is puzzling that the S1P dose response curves for stimulation of Rho-dependent FN deposition and G_i-dependent Erk1/2 activation are so different and both readouts are obtained in cells transfected with Edg-2. One explanation is that S1P is a ligand for Edg-2 and there is differential usage of G-proteins as a consequence of ligation of Edg-2 by LPA or S1P. LPA stimulation of Rho is mediated by $G_{12/13}$ [44]. The complexes that form between Edg-2 and low nanomolar concentrations of LPA or S1P, therefore, would be presumed to be equally effective in activating $G_{12/13}$. For activation of G_i, in contrast, the complex of Edg-2 and LPA would be much favored over the complex of Edg-2 and S1P. The other likely explanation is that transfection with Edg-2 sensitized cells to activation of the Edg-5 S1P receptor, which signals through G_i, G_q, and G_{13} [45].

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