Sphingosine 1-phosphate stimulation of the p42/p44 mitogen-activated protein kinase pathway in airway smooth muscle

Role of endothelial differentiation gene 1, c-Src tyrosine kinase and phosphoinositide 3-kinase

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We report here that cultured airway smooth muscle cells contain transcripts of endothelial differentiation gene 1 (EDG-1), a prototypical orphan G_i-coupled receptor whose natural ligand is sphingosine 1-phosphate (S1P). This is consistent with data that showed that S1P activated both c-Src and p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) in a pertussis toxin (PTX)-sensitive manner in these cells. An essential role for c-Src was confirmed by using the c-Src inhibitor, PP1, which markedly decreased p42/p44 MAPK activation. We have also shown that phosphoinositide 3-kinase (PI-3K) inhibitors (wortmannin and LY294002) decreased p42/p44 MAPK activation. An essential role for PI-3K was supported by experiments that showed that PI-3K activity was increased in Grb-2 immunoprecipitates from S1P-stimulated cells. Significantly, Grb-2 associated PI-3K activity was decreased by pretreatment of cells with PTX. Finally, we have shown that the co-stimulation of cells with platelet-derived growth factor (PDGF) and S1P (which failed to stimulate DNA synthesis) elicited a larger p42/p44 MAPK activation over a 30 min stimulation compared with each agonist alone. This was associated with a S1P-dependent increase in PDGF-stimulated DNA synthesis. These results demonstrate that S1P activates c-Src and Grb-2–PI-3K (intermediates in the p42/p44 MAPK cascade) via a PTX-sensitive mechanism. This action of S1P is consistent with the stimulation of EDG-1 receptors. S1P might also function as a co-mitogen with PDGF, producing a more robust activation of a common permissive signal transduction pathway linked to DNA synthesis.

Key words: lipid kinases, proliferation, Ras, sphingolipids.

INTRODUCTION

Sphingosine 1-phosphate (S1P) belongs to a group of platelet-derived lipid mediators that regulate cell differentiation, survival and proliferation in many cell types [1]. S1P binds to prototypical members of the orphan endothelial differentiation gene (EDG) family that include EDG-1, EDG-2/vzg-1, ARG16/H218 and EDG-3 [2–6], which are G_i-linked receptors. S1P has been shown to stimulate p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) via a pertussis toxin-sensitive pathway [7,8] in Swiss 3T3, airway smooth muscle (ASM) and transfected HEK 293 cells that overexpress EDG-1 [2]. The existence of novel S1P-specific receptors is further supported by reports from N1E-115 neuronal cells [9], human embryonic kidney cells [10], atrial myocytes [11] and aortic endothelial cells [12].

The mechanism used by S1P to activate the p42/p44 MAPK cascade has not been characterized. Other G_i-coupled receptors have been shown to regulate non-receptor tyrosine kinases, such as c-Src, which functions as an intermediate between $G_i\beta\gamma$ and Ras-dependent p42/p44 MAPK activation [13–15]. $G_i\beta\gamma$-dependent PI-3K (e.g. PI-3Kγ) also seems to participate in the regulation of a novel tyrosine kinase that is responsible for the phosphorylation of the Grb-2 adaptor protein, p100 [16], which is significant because Grb-2 is an intermediate in the p42/p44 MAPK cascade. Indeed, overexpression of PI-3Kγ induced the activation of p42/p44 MAPK in transfected COS cells [17]. Several G_i-coupled receptor agonists have also been implicated in the transactivation of growth factor receptors, which function as scaffolds for the recruitment of signalling proteins [18–21]. For example, the stimulation of vascular smooth-muscle cells by angiotensin II results in phosphorylation of the platelet-derived growth factor (PDGFβ) receptor [18], whereas thrombin triggers phosphorylation of the insulin-like growth factor 1 receptor in aortic smooth-muscle cells [19]. In rat-1 fibroblasts, the epidermal growth factor (EGF) receptor and p185^{neu} oncoproteins become phosphorylated on stimulation with endothelin-1, thrombin and lysophosphatidate [20,21].

Using cultured ASM cells, we have further investigated the regulation of the p42/p44 MAPK cascade by S1P. We have focused on the role of EDG receptors, c-Src tyrosine kinases and PI-3K. We have also assessed whether S1P- and PDGF-dependent signalling pathways interact to produce more efficient mitogenic stimulation of DNA synthesis.

MATERIALS AND METHODS

Materials

All biochemicals, including collagenase, elastase, soya bean trypsin inhibitor, EGF and PDGF-AB (specific for both PDGFα and PDGFβ receptors) were from Sigma Chemical Co. (Poole,

Abbreviations used: ASM, airway smooth muscle; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDG, endothelial differentiation gene; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; G_i/G_o, G-proteins; p42/p44 MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PI-3K, phosphoinositide 3-kinase; PTX, pertussis toxin; RT–PCR, reverse transcriptase-mediated PCR; S1P, sphingosine 1-phosphate.

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Dorset, U.K.). PP1, pertussis toxin (PTX) and tyrphostin AG1478 were from Calbiochem (Nottingham, Notts., U.K.). [^{3}H]Thymidine, [γ-^{32}P]ATP (3000 Ci/mol), MAPK Biotrak assay kits and enhanced chemiluminesence reagents were from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K). Cell culture supplies were from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). Antibodies against extracellular signal-regulated kinase 2 (ERK-2) were from Affiniti (Exeter, Devon, U.K.). D-*erythro*-S1P was from either Affiniti or TCS Biochemicals (Botolph Claydon, Bucks., U.K.). Anti-(c-Src) and anti-(Grb-2) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Reporter horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from the Scottish Antibody Production Unit (Carluke, Lanarkshire, Scotland, U.K.). Male Dunkin–Hartly guinea pigs (200–400 g) were used for the isolation of tracheal smooth muscle.

RNeasy total RNA isolation kit, QIA shredder, Qiaquick spin DNA kits were from Qiagen (Crawley, West Sussex, U.K.) Superscript II reverse transcriptase and primers were from Life Technologies; Dynazyme II DNA polymerase was from Flowgen (Shenstone, Staffs., U.K.); and DNase was from Promega (Southampton, Hants., U.K.). The AmpliTaq FS Dye terminator cycle sequencing kit was from PE-Applied Biosystems (Warrington, Cheshire, U.K.).

Cell culture

The preparation of the primary cultures of guinea-pig ASM cells has been described previously [22]. Their identity was confirmed to be smooth muscle by the presence of α-actin with the use of a smooth muscle-specific mouse anti-(α-actin) monoclonal antibody.

Total RNA extraction

RNeasy lysis buffer (600 μl) was added to cultured ASM cells and the lysate was passed through a QIAshredder. Total RNA was extracted in accordance with the RNeasy protocol instructions. This included incubating the extracted RNA with 4 units of DNase at 37 °C for 15 min to remove contaminating genomic DNA carried over during RNA purification.

Reverse transcriptase reaction

First-strand synthesis was performed with 5 μg of extracted total RNA and Superscript II reverse transcriptase. The reaction was primed with 500 ng of $(dT)_{18}$. This mixture was heated to 70 °C for 10 min and quickly chilled on ice. The total assay volume of the reverse transcriptase reaction was 20 μl and included (final concentrations) 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.03 mM dithiothreitol (DTT), 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM dTTP and 200 units of Superscript II reverse transcriptase. The mixture was incubated at 42 °C for 90 min and the reaction was terminated by being heated at 70 °C for 15 min.

PCR

A 100 μl PCR reaction mix contained 10 mM Tris/HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % (v/v) Triton X-100, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP and 2.5 units of Dynazyme II DNA polymerase together with 1 μM forward primer (TCC GCA AGA ACA TTT CCA AGG) and 1 μM reverse primer (GCT GCG GCT GAA TTC CAT G). The PCR reaction was performed with a Perkin Elmer 2400 Thermal Cycler with the following protocol: 95 °C for 5 min and 35 cycles of 95 °C for 1 min, 50 °C for 30 s and 65 °C for 1 min 40 s. This was followed by final extension for 10 min at 65 °C.

Sequence analysis

The product (15 μl) was subjected to electrophoresis on a 2 % (w/v) agarose gel and the PCR amplicon was excised and purified with a Pharmacia GFX gel extraction kit. The purified amplicon was sequenced in both directions on a PE-Applied Biosystems Division Model 373A automated DNA sequencer with the PCR primers and an AmpliTaq FS Dye terminator cycle sequencing kit.

p42/p44 MAPK assays

Confluent ASM cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 1 % (v/v) foetal calf serum and 1 % (v/v) donor horse serum for 24 h. Cells were stimulated with PDGF, EGF or S1P in the presence and the absence of inhibitors for the times indicated. In some experiments, cells were pretreated with PTX (0.1 μg/ml) for 24 h before stimulation with agonist.

p42 MAPK (ERK-2) phosphorylation assays

After stimulation with agonist, the medium was removed and boiling sample buffer [0.125 M Tris/HCl (pH 6.7)/0.5 mM $Na_4P_2O_7$/1.25 mM EDTA/2.5 % (v/v) glycerol/0.5 % SDS/ 25 mM DTT/1 % (w/v) Bromophenol Blue] was added. Cell lysates were harvested, subjected to SDS/PAGE and transferred to nitrocellulose sheets. p42 MAPK activation was detected by a shift in its electrophoretic mobility on immunoblots probed with anti-(ERK-2) antibody.

p42/p44 MAPK activity

Assays utilized a specific p42/p44 MAPK peptide substrate ($EGFR^{661-680}$ peptide synthesized to contain one phosphorylation site). Cell lysates were prepared by the addition of 0.5 ml of buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Cells were harvested in this buffer and homogenized by repeatedly (three times) passing them through a 0.24 mm gauge syringe needle. Each assay contained 5–10 μg of cell lysate protein and 0.5 μCi of [γ-^{32}P]ATP (final concentration 0.2 mM). Assays were performed under conditions in which less than 2 % of the substrate was utilized and in which product formation was linearly related to time. Assays were terminated by the addition of stopping solution. Samples were then spotted on peptide binding papers and washed three times with 1 % (v/v) acetic acid and twice with distilled water. The amount of ^{32}P-labelled peptide on each disc was quantified by liquid-scintillation counting.

Immunoprecipitation

After stimulation with agonist, the medium was removed and cells were lysed in 1 ml of ice-cold immunoprecipitation buffer [20 mM Tris/HCl/137 mM NaCl/2.7 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/1 % (v/v) Nonidet P40/10 % (v/v) glycerol/1 mg/ml BSA/0.5 mM sodium orthovanadate/0.2 mM PMSF/10 μg/ml leupeptin/10 μg/ml antipain/10 μg/ml pepstatin/10 μg/ml aprotinin (pH 8)] for 10 min at 4 °C. The material was harvested, centrifuged at 22000 $\boldsymbol{g}$ for 5 min at 4 °C, then

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200 μl of cell lysate supernatant (equalized for protein, 0.5–1 mg/ml) taken for immunoprecipitation with antibodies [5 μg of either anti-(c-Src) or anti-(Grb-2) together with 40 μl of 1 part immunoprecipitation buffer and 1 part Protein A/Protein G–Sepharose CL4B]. After agitation for 2 h at 4 °C, the immune complex was collected by centrifugation at 22000 ***g*** for 15 s at 4 °C. For c-Src, the beads were washed twice with buffer A [150 mM NaCl/10 mM sodium phosphate (pH 7)/2 mM EDTA/1 % (w/v) deoxycholate/1 % (v/v) Nonidet P40/0.1 % SDS/0.2 mM PMSF/10 μg/ml leupeptin/20 μg/ml aprotinin/ 50 mM NaF/2 mM sodium orthovanadate/0.1 % (v/v) mercaptoethanol] and then twice with buffer containing 10 mM Hepes, pH 7, 100 mM NaCl, 0.2 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin and 0.5 % (v/v) Nonidet P40 and once in buffer without Nonidet P40. For Grb-2, the beads were washed as above but with the omission of buffer A. c-Src immunoprecipitates were resuspended in buffer A for c-Src assays, whereas Grb-2 immunoprecipitates were resuspended in 20 mM β-glycerophosphate/5 mM $Na_4P_2O_7$/30 mM NaCl/1 mM DTT (pH 7.2) for PI-3K activity assays.

Grb-2 and c-Src immunoprecipitates were also resuspended in boiling sample buffer [0.125 M Tris/HCl (pH 6.7)/0.5 mM $Na_4P_2O_7$/1.25 mM EDTA/2.5 % (v/v) glycerol/0.5 % SDS/ 25 mM DTT/1 % (w/v) Bromophenol Blue] and taken for immunoblotting with anti-(Grb-2) and anti-(c-Src) antibodies.

Blotting

After transfer of proteins (immunoprecipitates and cell lysate samples) from SDS/PAGE to nitrocellulose, the sheets were blocked in 3 % (w/v) BSA in 10 mM Tris/HCl (pH 8)/0.15 M NaCl (TBS) for 1 h at 23 °C. These were then probed with anti-(ERK-2), anti-(c-Src) or anti-(Grb-2) antibodies in TBS containing 1 % (w/v) BSA and 0.05 % (v/v) Tween 20 for 12 h at 23 °C. After this time, the nitrocellulose sheets were washed in TBS containing 0.05 % (v/v) Tween 20. Detection of immunoreaction was by incubating nitrocellulose for 2 h at 23 °C with reporter HRP-linked anti-mouse or anti-rabbit antibodies in TBS containing 1 % (w/v) BSA and 0.05 % (v/v) Tween 20. After the blots had been washed as described above (to remove excess reporter antibody) immunoreactive bands were detected with the enhanced chemiluminesence detection kit.

c-Src assay

c-Src immunoprecipitates were incubated with 1.6 μg of acid-denatured rabbit muscle enolase (Sigma), 20 mM Hepes, pH 7, 10 mM $MnCl_2$, 20 μg/ml aprotinin and 5 μCi of [γ-^{32}P]ATP (3 μM) in a total reaction volume of 20 μl. The reaction was stopped by adding 2 × sample buffer containing 10 mM EDTA, 4 % (w/v) SDS, 5.6 M 2-mercaptoethanol, 20 % (v/v) glycerol, 200 mM Tris/HCl, pH 6.8, and 1 % (w/v) Bromophenol Blue. Samples were then boiled for 2 min, cooled and subjected to SDS/PAGE.

PI-3K assay

Resuspended Grb-2 immunoprecipitates (40 μl) were each combined with 20 μl of PtdIns (3 mg/ml) in incubation buffer containing 1 % (w/v) cholate. To each, 40 μl of [^{32}P]ATP (containing 3 μM Na_2ATP, 7.5 mM $MgCl_2$ and 0.25 mCi/ml [^{32}P]ATP) was added. The reaction was performed at 37 °C for 15 min and was terminated by the addition of 450 μl of chloroform/methanol (1:2, v/v). The organic and aqueous phases were resolved by the addition of 150 μl of chloroform and 150 μl of 1 M HCl. Samples were mixed and centrifuged at 4200 ***g*** for 10 min. This was repeated and the lower phase was harvested, evaporated to dryness and [^{32}P]PtdIns3*P* was resolved by TLC with chloroform/methanol/ammonia/water (14:20:3:5, by vol.) in parallel with a non-radioactive standard. Radioactive bands were detected by autoradiography and samples corresponding to [^{32}P]PtdIns3*P* were scraped from the plate and subjected to Čerenkov counting.

Uptake of [^{3}H]thymidine

Cells (70–80 % confluent) in 24-well plates were placed in DMEM containing 0.1 % (v/v) foetal calf serum and 0.1 % (v/v) donor horse serum for 24 h at 37 °C. This was replaced with DMEM and incubated for a further 20 h at 37 °C in the presence and the absence of either PDGF, S1P or both. [^{3}H]Thymidine (0.25 μCi per 2.5×10^5 cells) was added to incubations for a further 5 h. Incubations were terminated by washing each well in 1 ml of ice-cold PBS containing 10 mM K_2HPO_4, 10 mM KH_2PO_4, pH 7.4, and 150 mM NaCl. The medium was aspirated, the cells were washed three times (10 min each) with 1 ml of 10 % (w/v) trichloroacetic acid, and nuclear material was dissolved in 0.25 ml of 0.1 % SDS/0.3 M NaOH. [^{3}H]Thymidine uptake was quantified by liquid-scintillation counting.

RESULTS

S1P receptor-dependent activation of p42/p44 MAPK

Figure 1(a) shows that S1P activates p42/p44 MAPK in a concentration-dependent manner, which saturates at 5 μM S1P. The initial rate of p42/p44 MAPK activation was faster with 50 μM S1P than with 5 μM (Figures 1b and 1c). These results are consistent with a simple receptor-binding phenomenon. Furthermore PTX (0.1 μg/ml, 24 h), which catalyses the ADP-ribosylation of G_i (G_o is not expressed in ASM cells [23]) and uncouples G-protein linked receptors from their effectors, abolished the p42/p44 MAPK activation at supramaximal S1P (Table 1). The effect of PTX was associated with the complete endogenous ADP-ribosylation of G_i, as evidenced from experiments that showed that PTX failed to catalyse [^{32}P]ADP-ribosylation of $G_i\alpha$ in membranes prepared from PTX (0.1 μg/ml, 24 h)-treated cells [24].

EDG-1, whose natural ligand is S1P, is a prototypical member of a G-protein-coupled receptor family [2–6]. Reverse transcriptase-mediated PCR (RT–PCR) with specific primers for EDG-1 amplified a 347 bp product from total ASM RNA (Figure 1d). The deduced amino acid sequence of the PCR product was more than 93 % similar to the corresponding region in human, rat and mouse EDG-1 (Figure 1e). The amplicon was not obtained when reverse transcriptase was omitted from the first-strand DNA synthesis (results not shown). RT–PCR with specific primers for EDG-3, which also binds S1P, did not amplify a product (results not shown).

Growth factor receptor transactivation

Several G_i-coupled receptors have been implicated in growth factor receptor transactivation. The best-characterized ligand is LPA, which binds to the EDG-2 receptor [6] and transactivates the EGF receptor (EGFR), an intermediate step in a signal transduction pathway linked to p42/p44 MAPK activation in some cell types [20,21]. We therefore investigated the possibility that S1P, possibly acting at EDG-1 receptors, might also induce EGFR transactivation. The EGFR kinase inhibitor, tyrphostin AG1478, which blocked the EGF-dependent activation of

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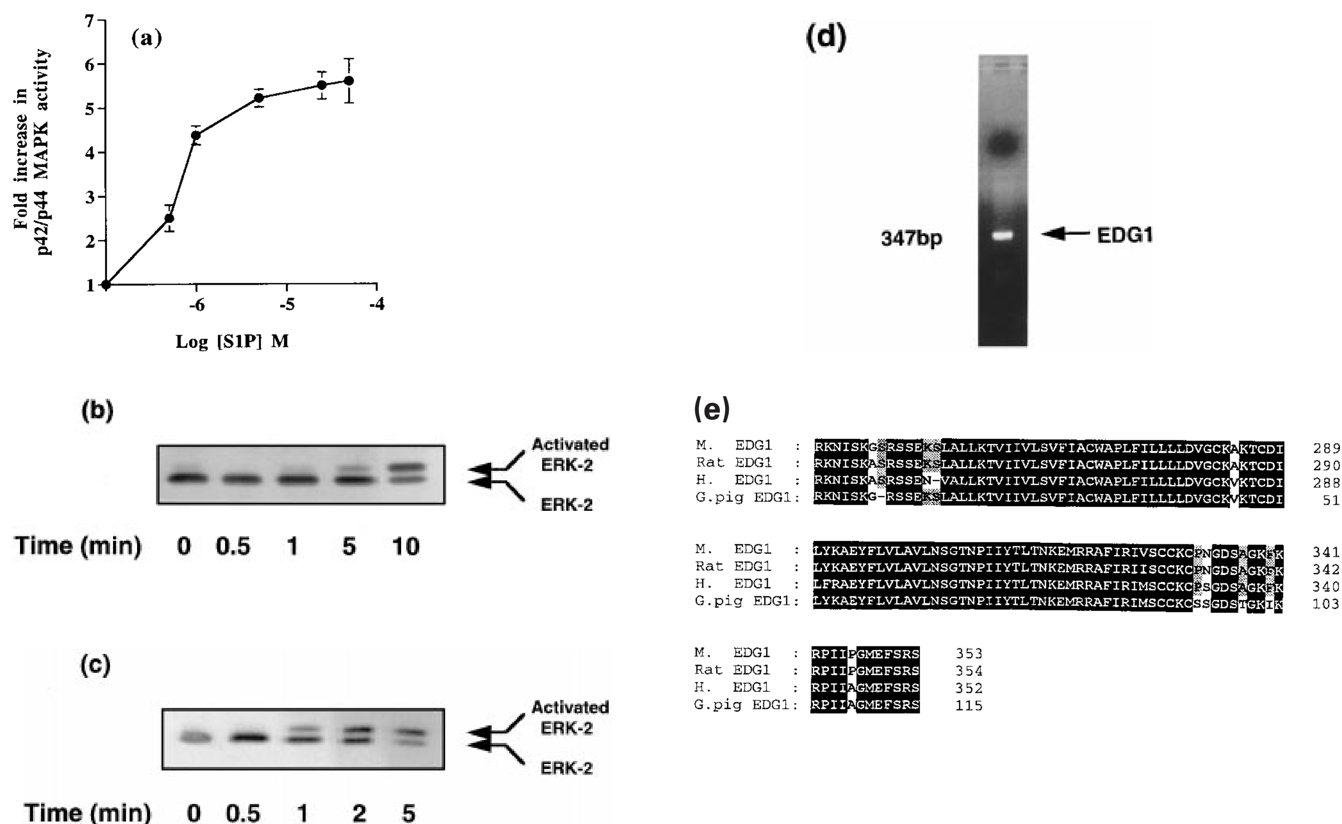

Figure 1 EDG-1 expression and p42/p44 MAPK activation

(**a**) ASM cells were stimulated with various concentrations of S1P for 10 min; p42/p44 MAPK activity was measured in cell lysates by using a specific peptide assay kit. Basal p42/p44 MAPK activity in control cells was 140 ± 13 pmol/min per mg of protein. Results are expressed as fold increases over basal p42/p44 MAPK activity in control cells (means $\pm$ S.D. for $n = 3$ separate cell preparations). (**b**) ASM cells were stimulated with 5 μM S1P for various times. (**c**) ASM cells were stimulated with 50 μM S1P for various times. (**b**, **c**) are shift blots showing p42 MAPK/ERK-2 phosphorylation and are representative results of experiments performed on three separate cell preparations. ERK-2 and activated ERK-2 are denoted by arrows. (**d**) ASM total RNA was used in an RT–PCR reaction to detect EDG-1 receptor transcripts. Primers were designed to amplify a 347 bp product corresponding to a unique region of the EDG-1 cDNA. (**e**) The amino-acid sequence alignment of guinea-pig (G.pig) EDG-1 with the corresponding human (H.), mouse (M.) and rat EDG-1. Dark shading shows amino acid similarity between guinea-pig, human, mouse and rat EDG-1.

Table 1 Effect of PTX and inhibitors on the S1P-dependent activation of p42/p44 MAPK

ASM cells were treated with or without PTX (0.1 μg/ml) for 24 h. These cells were then incubated with or without PP1 (10 μM) or wortmannin (50 nM) or LY294002 (50 μM) for 15 min before stimulation with S1P (50 μM) for 10 min. Similar results were obtained with 5 μM S1P. p42/p44 MAPK activity was measured in cell lysates by using a specific peptide assay kit. Basal p42/p44 MAPK activity in control cells was 115 ± 23 pmol/min per mg of protein. Results are expressed as fold increases over basal p42/p44 MAPK activity in control cells (means $\pm$ S.D. for $n = 5$–10 separate cell preparations). The inhibitor of MEK-1 activation, PD098059, abolished p42/p44 MAPK activation in response to S1P (results not shown). $^{*}P < 0.001$ compared with S1P in the absence of inhibitors or PTX (Student's *t* test).

Treatment	Pretreatment with PTX	Fold activation
Control	−	1 ± 0.2
S1P	−	6.1 ± 0.6
Wortmannin	−	0.8 ± 0.05
Wortmannin + S1P	−	$0.95 \pm 0.1^{*}$
LY294002	−	0.8 ± 0.02
LY294002 + S1P	−	$3.5 \pm 0.5^{*}$
PP1	−	0.9 ± 0.1
PP1 + S1P	−	$2.2 \pm 0.5^{*}$
Control	+	0.8 ± 0.2
S1P	+	$1.4 \pm 0.3^{*}$

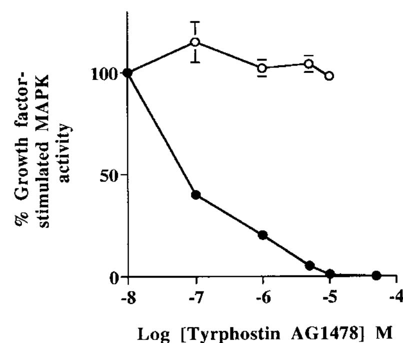

Figure 2 Effect of tyrphostin AG1478 on p42/p44 MAPK activation

ASM cells were treated with or without various concentrations of tyrphostin AG1478 for 15 min before stimulation with either S1P (5 μM) (○) or EGF (100 nM) (●) for 10 min; p42/p44 MAPK activity was measured in cell lysates by using a specific peptide assay kit. Growth-factor-stimulated p42/p44 MAPK activity was calculated by subtracting the corresponding basal activity. Results are expressed as percentage inhibitions of growth-factor-stimulated p42/p44 MAPK activity (100%) and are means $\pm$ S.D. for $n = 4$ separate cell preparations.

p42/p44 MAPK ($IC_{50} < 100$ nM) failed to inhibit the S1P-induced response (Figure 2); thus EGFR transactivation by S1P can be rejected.

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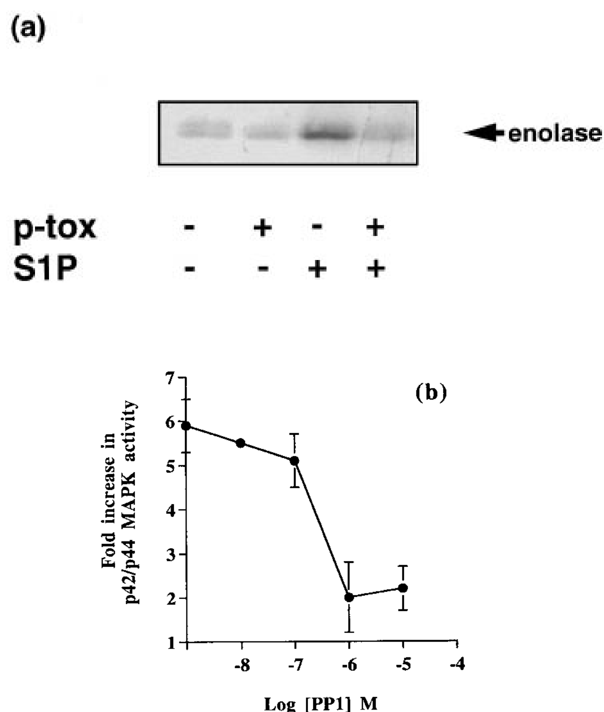

Figure 3 c-Src activation

(**a**) Autoradiograph showing c-Src activation in cells treated with or without PTX (p-tox) (0.1 μg/ml) for 24 h before stimulation with S1P (5 μM, 10 min). c-Src activity in anti-c-Src immunoprecipitates was measured with purified enolase and [^{32}P]ATP as substrates. ^{32}P-labelled enolase was then resolved by SDS/PAGE. Immunoprecipitates were also immunoblotted with anti-(c-Src) antibody to show that equal amounts of c-Src had been immunoprecipitated from each sample (results not shown). This is a representative result of an experiment performed on three separate cell preparations. (**b**) ASM cells were pretreated with various concentrations of PP1 for 15 min before stimulation with S1P (50 μM) for 10 min; p42/p44 MAPK activity was measured in cell lysates by using a specific peptide assay kit. Basal p42/p44 MAPK activity in control cells was 115 $\pm$ 23 pmol/min per mg of protein. Results are expressed as fold increases over basal p42/p44 MAPK activity in control cells (means $\pm$ S.D. for $n = 5$–10 separate cell preparations). PP1 was without significant effect on basal p42/p44 MAPK activity (results not shown).

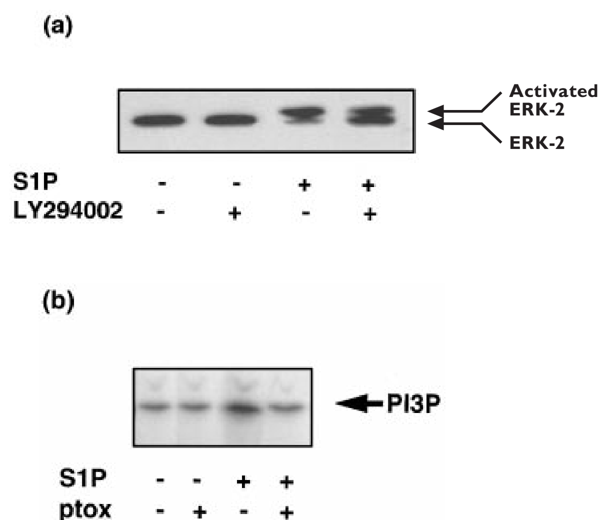

Figure 4 Role of PI-3K

(**a**) Shift blot showing p42 MAPK/ERK-2 phosphorylation in cells treated with or without LY294002 (50 μM) for 15 min before stimulation with S1P (50 μM, 10 min). ERK-2 and activated ERK-2 are denoted by arrows. (**b**) Autoradiograph showing PI-3K activity in Grb-2 immunoprecipitates from cells pretreated with or without PTX (ptox) (0.1 μg/ml) for 24 h before stimulation with S1P (5 μM) for 10 min. These are representative results of experiments performed on three separate cell preparations. PI3P, PtdIns3P.

The role of c-Src and PI-3K

Several authors have reported that c-Src tyrosine kinases function as intermediates between G_i-coupled receptors and the Ras/p42/p44 MAPK pathway [13–15]. Consistent with this model, S1P induced rapid c-Src activation, which was abolished by PTX (0.1 μg/ml, 24 h) (Figure 3a). An essential role for c-Src was confirmed by using the c-Src inhibitor PP1. PP1 (10 nM to 10 μM) inhibited p42/p44 MAPK activation in a concentration-dependent manner at supramaximal S1P concentrations (Table 1 and Figure 3b) (10 μM PP1 also inhibited EGFR- and LPA-dependent p42/p44 MAPK activation in transfected COS-7 cells [21]).

These results show that the S1P-induced activation of c-Src and p42/p44 MAPK and inhibition by PTX and PP1 are correlated.

Two structurally dissimilar PI-3K inhibitors [wortmannin (50 nM) and LY294002 (50 μM)] were also shown to decrease p42/p44 MAPK activation at supramaximal S1P concentrations (Table 1 and Figure 4a). Wortmannin was more effective than LY294002 against p42/p44 MAPK, suggesting that the former might be a better PI-3K inhibitor or that it might act at additional sites in the cascade. An essential role for PI-3K was supported by experiments that showed that PI-3K activity was increased in Grb-2 immunoprecipitates (Grb-2 is an intermediate in the p42/p44 MAPK cascade) from S1P-stimulated cells. Significantly, the Grb-2 associated PI-3K activity was decreased by pretreating cells with PTX (Figure 4b). PI-3K and Grb-2 were not present in immunoprecipitates when the Grb-2 antibody was omitted from the immunoprecipitation protocol (results not shown).

These results suggest that S1P might promote Grb-2–PI-3K complex formation, which might be an intermediate step in the signal transduction pathway linked to p42/p44 MAPK activation.

Co-mitogenic signalling

We measured [^{3}H]thymidine uptake to investigate whether S1P and PDGF stimulate DNA synthesis in ASM cells. S1P (5 μM) failed to stimulate [^{3}H]thymidine uptake, whereas a maximal concentration of PDGF (10 ng/ml) stimulated an approx. 15-fold increase in [^{3}H]thymidine uptake. Combined stimulation with both S1P and maximal PDGF stimulated an approx. 19-fold increase in [^{3}H]thymidine uptake (control, 3841 $\pm$ 694 d.p.m.; PDGF, 58797 $\pm$ 5874 d.p.m.; S1P, 3898 $\pm$ 795 d.p.m., PDGF/S1P, 72101 $\pm$ 4394 d.p.m.; means $\pm$ S.D. for $n = 3$ separate cell preparations; for PDGF/S1P, $P < 0.05$ compared with PDGF alone).

Figures 5(a) and 5(b) show that the co-stimulation of cells with PDGF (10 ng/ml) and S1P (5 μM) induced a larger p42/p44 MAPK activation between 0 and 5 min than with either agonist alone. The activation saturated to a level seen with PDGF at 10 min and then declined, remaining well above basal, during the following 20 min. The combined p42/p44 MAPK activation was larger at 20 and 30 min compared with each agonist alone and this was associated with a S1P-dependent increase in PDGF-stimulated [^{3}H]thymidine uptake (see above).

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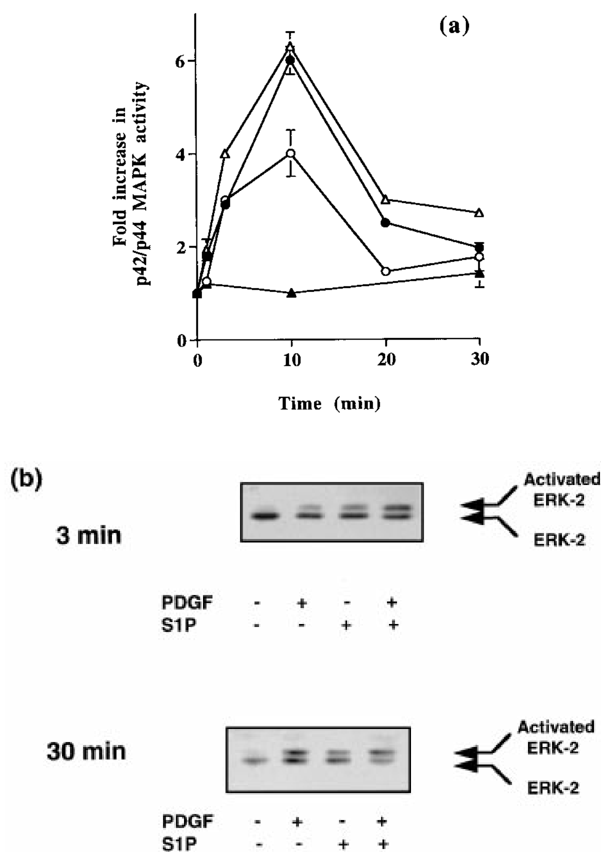

Figure 5 p42/p44 MAPK activation in response to S1P and PDGF

Cells were either untreated (▲) or treated with either S1P (5 μM, ○) or PDGF (10 ng/ml, ●) or both (△) for various times. (**a**) p42/p44 MAPK activity was measured in cell lysates by using a specific peptide assay kit. The basal p42/p44 MAPK activity in control cells was 125 ± 10 pmol/min per mg of protein. p42/p44 MAPK activities are expressed as fold increases over basal p42/p44 MAPK activity in control cells (means $\pm$ S.D. for $n = 3$ separate cell preparations). (**b**) Shift blot showing p42 MAPK/ERK-2 phosphorylation in cells treated with either PDGF (10 ng/ml), S1P (5 μM) or both for 3 min (upper panel) and 30 min (lower panel). ERK-2 and activated ERK-2 are denoted by arrows. The shift blots are representative results of experiments performed on three separate cell preparations.

DISCUSSION

We have demonstrated here that the S1P-dependent activation of p42/p44 MAPK displays characteristics typical of a G_i-coupled receptor-mediated event. The response was concentration-dependent and was inhibited by PTX (which was used to inactivate G_i). Furthermore EDG-1 receptor transcripts (a prototypical member of an orphan receptor subfamily composed of EDG-2/vzg-1, ARG16/H218 and EDG-3), for which S1P is the natural ligand [2–6], were identified in ASM cells. Taken together, the results are consistent with a model in which the S1P-dependent activation of p42/p44 MAPK is mediated by a PTX-sensitive G-protein-coupled receptor, possibly EDG-1. Previous studies have demonstrated that EDG receptor signalling bifurcates down heterotrimeric G_i-dependent (PTX-sensitive) and Rho-dependent (PTX-insensitive) pathways [2,4]. Heterotrimeric G-protein-mediated responses include Ca^{2+} mobilization, inhibition of forskolin-stimulated cAMP formation and p42/p44 MAPK activation. Rho-mediated responses include exaggerated cell–cell aggregation, enhanced expression of cadherins and the formation of well-developed adheren (e.g. focal adhesion) junctions [2].

We have also shown here, that S1P activates c-Src tyrosine kinases via a PTX-sensitive mechanism. This is consistent with other reports that c-Src has an essential role in the regulation of the p42/p44 MAPK cascade by G_i-coupled receptors [13–15]. On the basis of the present results and those from other papers, we suggest that S1P might promote the generation of free $G_i\beta\gamma$ subunits, which stimulate c-Src directly. Alternatively, $G_i\beta\gamma$ subunits might act as adaptor proteins, locating c-Src in a complex with intermediate activating proteins, e.g. other non-receptor tyrosine kinases. The adaptor model is based on evidence that G-protein-coupled receptors use G-protein $\beta\gamma$ subunits to recruit G-protein-coupled receptor kinases [25].

An essential role for PI-3K was confirmed by experiments showing that two structurally dissimilar PI-3K inhibitors decreased the activation of p42/p44 MAPK by S1P. We have also found that S1P promotes Grb-2–PI-3K complex formation via a PTX-sensitive mechanism. Certain PI-3K isoforms (e.g. PI-3Kγ) are directly activated by $G_i\beta\gamma$ [17]; this might be an important step in the formation of the Grb-2–PI-3K complex. The nature of the interaction between PI-3K and Grb-2 has not been identified. Future studies are necessary to characterize this interaction, i.e. the role of $G_i\beta\gamma$ subunits, Grb-2 SH2 and SH3 domains, tyrosine phosphorylation intermediates and PH domains in PI-3K.

Co-stimulation with S1P and PDGF elicited a larger p42/p44 MAPK activation over 30 min than with each agonist alone. This was associated with a S1P-dependent increase in PDGF-stimulated DNA synthesis. These results suggest that the magnitude of DNA synthesis might be governed by various 'threshold' levels of p42/p44 MAPK activation throughout the 30 min stimulation. The extent to which these levels are breached at each time point might explain why combined stimulation with PDGF/S1P produced more DNA synthesis than stimulation with PDGF. S1P produced a much weaker p42/p44 MAPK activation, which is probably below these 'threshold' levels and is therefore non-mitogenic.

Conclusion

S1P is a novel extracellular lysophospholipid mediator that binds to a G_i-coupled receptor (possibly the EDG-1 receptor) to regulate c-Src and Grb-2–PI-3K, which are intermediates in the p42/p44 MAPK cascade. S1P might also function as a co-mitogen with growth factors, producing a more robust activation of a common permissive signal transduction pathway linked to DNA synthesis.

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