Lysophosphatidic acid prevents apoptosis in fibroblasts via G_i-protein-mediated activation of mitogen-activated protein kinase

Xianjun FANG*, Shuangxing YU*, Ruth LAPUSHIN*, Yiling LU*, Tatsuro FURUI*, Linda Z. PENN†, David STOKOE‡, James R. ERICKSON§, Robert C. BAST, JR* and Gordon B. MILLS*[1]

*Department of Molecular Therapeutics, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Box 317, Houston, TX 77030, U.S.A., †Department of Molecular and Medical Genetics, Ontario Cancer Institute and University of Toronto, Toronto, Ontario, Canada M5G 2M9, ‡Cancer Research Institute, University of California at San Francisco, San Francisco, CA 94143, U.S.A., and §Atairgin Technologies, Inc., 4 Jenner, Irvine, CA 92618, U.S.A.

Lysophosphatidic acid (LPA) is a naturally occurring phospholipid with multiple biological functions. In the present study, we demonstrate that, besides its mitogenic activity, LPA is a potent survival factor, preventing serum-deprivation-induced apoptosis in fibroblasts and other cell types. Both the proliferative effect and survival activity of LPA are sensitive to the action of pertussis toxin (PTX), indicating that both processes are mediated by G_i protein(s). We therefore focused on the role of G_i-protein-mediated signalling events in the promotion of cell survival by LPA. In addition to activation of mitogen-activated protein kinase (MAPK), LPA stimulates a modest PTX-sensitive phosphorylation/activation of the serine/threonine kinase Akt, a survival mediator downstream of phosphoinositide 3-kinase (PI3K). Inhibition of PI3K with LY 294002 or wortmannin resulted in a marked inhibition of LPA-induced DNA synthesis, and yet the survival activity of LPA decreased by only 20–30 %, suggesting a limited input of the PI3K–Akt cascade in LPA-induced cell survival. In contrast, inhibition of MAPK activation by the MEK-1 inhibitor, PD 98059, blocked both the proliferative and survival effects of LPA. These results indicate that LPA promotes cell survival largely via G_i-protein-mediated activation of ERK1/ERK2, or other PD 98059-sensitive member(s) of the MAPK family.

Key words: cell survival, cell proliferation, extracellular-signal-regulated kinase (ERK), NIH 3T3 cells, phosphatidylinositol 3-kinase.

INTRODUCTION

Lysophosphatidic acid (LPA; 1-acyl-2-lyso-*sn*-glycero-3-phosphate) is a natural glycerophospholipid, which possesses a pleiotropic range of biological actions. Although not present at significant concentrations in plasma or freshly isolated blood, LPA is generated and released during blood clotting, and is therefore considered to be a mediator in wound healing and tissue regeneration [1–3]. The most prominent effects of LPA include stimulation of cell proliferation [4], platelet aggregation [5], smooth-muscle cell contraction [6] and tumour cell invasion [7]. As a normal constituent of serum, LPA contributes to its mitogenic activity and has been identified as the factor responsible for serum-induced focal-adhesion assembly and stress-fibre formation in cultured fibroblasts [8]. LPA is present at elevated levels in ascites and plasma of ovarian cancer patients, and may thus contribute to the progression of certain types of human cancer [9,10].

LPA interacts with specific G-protein-coupled receptors on the cell membrane. Several candidate genes (*Edg*-2/*Vzg*-1, *Edg*-4 and *Edg*-7) encoding mammalian receptors for LPA have been described previously [11]. Most cell types in culture express more than one Edg receptor and respond to LPA, making it difficult to characterize the receptor-dependence of a particular response to LPA. A number of G-protein-dependent signalling cascades have been identified as potentially mediating the action of LPA, e.g. stimulation of phospholipases C and D [4,12,13], inhibition of adenylate cyclase [4], activation of Ras and the downstream mitogen-activated protein kinase (MAPK) [14], and tyrosine phosphorylation of focal-adhesion proteins via a Rho-dependent pathway [15].

We have demonstrated previously that, compatible with its growth-factor-like activity, LPA reduces the sensitivity of ovarian cancer cell lines to apoptosis induced by the chemotherapeutic agent cisplatin, implying an anti-apoptotic role for LPA [16]. Others have recently reported that LPA acts as a survival factor suppressing serum-starvation-induced apoptosis in cultured renal proximal tubular cells [17], macrophages [18], T cells [19] and Schwann cells [20]. In contrast, LPA has also been shown to induce apoptosis in hippocampal neurons [21] and nerve-growth-factor-differentiated PC12 cells [22]. These studies underscore the involvement of the natural phospholipid LPA in regulation of cell viability *in vitro* and *in vivo*. However, this novel facet of LPA function is not well understood, particularly compared with LPA's mitogenic effect.

In the present study, we have evaluated the role of LPA in the regulation of cell survival-versus-proliferation in fibroblast cell lines. Besides its mitogenic activity, LPA is a potent survival factor in fibroblasts, inhibiting serum-deprivation-induced apoptosis. Both the proliferative response and survival effect of LPA are sensitive to pertussis toxin (PTX), indicating a critical role for G_i proteins in the delivery of LPA's growth and survival signals. Thus we focused on the role of LPA-activated signal pathways downstream of G_i proteins in the proliferative and survival responses to LPA. Our results indicate that G_i-protein-mediated activation of MAPK/extracellular-signal-regulated kinase (ERK) is required for the majority of the survival activity of LPA.

Abbreviations used: BAD, **B**cl-2/Bcl-X_L-**a**ntagonist, causing cell **d**eath; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PTX, pertussis toxin.

[1] To whom correspondence should be addressed (e-mail gmills@notes.mdacc.tmc.edu).

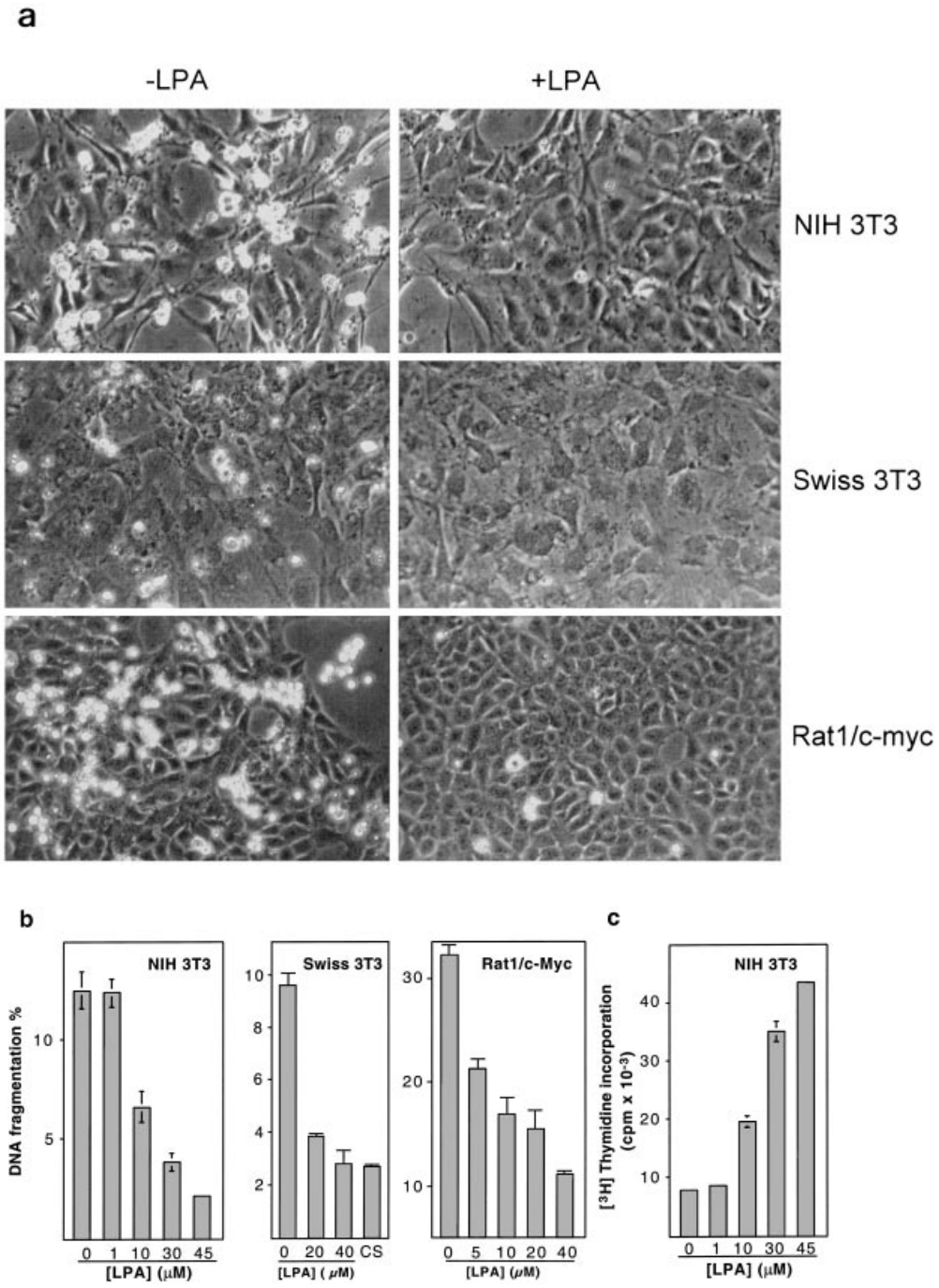

Figure 1 LPA inhibits serum-deprivation-induced apoptosis in NIH 3T3, Swiss 3T3 and Rat1/c-Myc cells

(**a**) Phase-contrast microscopy of NIH 3T3, Swiss 3T3 and Rat1/c-Myc cells incubated in serum-free medium for 16–18 h with vehicle (−LPA) or with 20 μM LPA (+LPA) ($\times$200 magnification). (**b**) Quantitative analysis of the survival activity of LPA. The cells were incubated in serum-free medium in the presence of the indicated concentrations of LPA or 5% calf serum. The cells were harvested after 16–18 h of incubation. Fragmented and unfragmented DNA was separated and analysed as detailed in the Experimental section. NIH 3T3 and Swiss 3T3 cells were pre-labelled with [^{3}H]thymidine. The radioactivity within each fraction (fragmented and unfragmented) of DNA was measured by scintillation counting. For Rat1/c-Myc cells, percentages of DNA fragmentation were determined by measuring adenosine contents of fragmented and intact DNA. (**c**) The proliferative effect of different concentrations of LPA in NIH 3T3 cells. The activity was measured by [^{3}H]thymidine incorporation as described in the Experimental section. For both (**b**) and (**c**), the results represent means $\pm$ S.D. for triplicate assays in a single experiment. Consistent results were obtained from three independent experiments.

EXPERIMENTAL

Reagents

LPA (oleoyl; $C_{18:1}$) was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.) or Sigma (St Louis, MO, U.S.A.). Fetal-bovine serum, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), wortmannin and anti-(β-actin) monoclonal antibody were obtained from Sigma. Pharmaceutical inhibitors (PTX, LY 294002 and PD 98059) were purchased from Calbiochem (San Diego, CA, U.S.A.). Fatty-acid-free BSA was

from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Antibodies raised against Akt, phospho-Akt (Ser-473), p70 S6 kinase (p70 S6K), and phospho-p70 S6K (Thr-389) were from New England Biolabs (Beverly, MA, U.S.A.). Anti-phospho-ERK1/ERK2 antibody was from Promega (Madison, WI, U.S.A.). Other antibodies used in the study were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). [*methyl*-^{3}H]Thymidine (5.0 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, U.S.A.).

Cells

NIH 3T3, Swiss 3T3, HeLa, OVCAR-3 and Jurkat cells were obtained from A. T. C. C. (Rockville, MD, U.S.A.), and cultured following the manufacturer's recommendations. Sources and maintenance of Rat-1 and Rat1/c-Myc cell lines have been described previously [4,23]. All these cell lines were frozen at early passages, and used for less than 10 weeks in continuous culture.

Mitogenic assay

Growth-factor-stimulated cell proliferation was measured by [^{3}H]thymidine incorporation. LPA was freshly prepared by dissolving in PBS containing 1% (w/v) fatty-acid-free BSA. NIH 3T3 cells were plated at $(2.5–3.0) \times 10^5$/60-mm dish. After three days, when dishes were at approx. 70–80% confluence, the cells were washed and fed with serum-free medium. After 16 h of incubation with LPA or vehicle, cells were pulse-labelled with [^{3}H]thymidine (2 μCi/dish) for the final 6 h. The cells were washed twice with PBS, twice with 5% (w/v) trichloroacetic acid and once with 95% (v/v) ethanol. Trichloroacetic-acid-insoluble material was dissolved in 0.2 M sodium hydroxide overnight, and then scintillation-counted for radioactivity.

Apoptotic assay

In 3T3 and Rat1/c-Myc cells, percentages of DNA fragmentation were determined by isolating and measuring fragmented DNA as against intact DNA. In brief, the two fractions of DNA were separated by lysing cells in TE buffer (10 mM Tris/HCl, pH 7.4/1 mM EDTA) containing 0.2% (v/v) Triton X-100, followed by centrifugation for 15 min (15000 ***g***). The fragmented soluble DNA was released to the supernatant, whereas the intact DNA remained in the pellet. The two fractions of DNA were quantified by either scintillation counting (if the cells were prelabelled with [^{3}H]thymidine) or measuring the content of adenosine using a spectrometric technique, as described by Duke et al. [24]. The percentage of DNA fragmentation was calculated according to the following equation: % DNA fragmentation = {[Radioactivity (c.p.m.) or measurement of adenosine at A_{600} in the supernatant]/[Radioactivity (c.p.m.) or measurement of adenosine at A_{600} in the supernatant + radioactivity (c.p.m.) or measurement of adenosine at A_{600} in the pellet]} $\times$ 100

Biochemical assays

Expression and activation of protein kinases were assessed by Western blotting using antibodies raised against individual kinases or phospho-specific antibodies that react with phosphorylated/activated kinases only. Lysates were made from control and stimulated cells in SDS sample buffer or ice-cold Triton X-100 lysis buffer [25]. Equal amounts of total cellular protein were separated by SDS/PAGE, transferred to immobilon (PVDF), and immunoblotted with antibodies following the protocols provided by manufacturers. Immunocomplexes were detected by an enhanced chemiluminescence detection kit (Amersham) using horseradish-peroxidase-conjugated secondary antibodies.

RESULTS

LPA protects cells from serum-starvation-induced apoptosis

LPA acts as a strong mitogen towards many fibroblast lines. In particular, 3T3 and Rat-1 cells have been those used most commonly for the study of LPA-mediated mitogenesis [1,4]. To explore the possibility that LPA might also function as a survival factor in fibroblasts, we examined the effect of LPA on growth-factor-withdrawal-induced apoptosis. Consistent with previous reports, NIH 3T3 and Swiss 3T3 cells undergo apoptosis upon serum deprivation [26–28]. In both cell lines, supple-

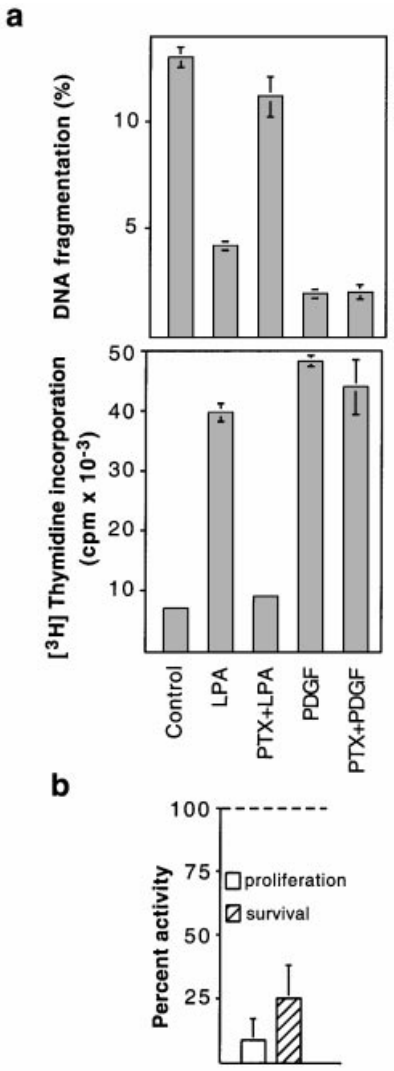

Figure 2 PTX inhibits LPA-mediated cell proliferation and survival

NIH 3T3 cells were incubated in serum-free medium with LPA (30 μM) or PDGF B/B (20 ng/ml) in the presence or absence of PTX (25 ng/ml). PTX was added at least 5 h before LPA or PDGF. (**a**) Cell proliferation was measured by [^{3}H]thymidine incorporation, as described in the Experimental section. Apoptosis was analysed in parallel by measuring cellular DNA fragmentation. [^{3}H]Thymidine-pre-labelled cells were incubated with LPA, PDGF or vehicle for 16–18 h, before harvesting for apoptotic analysis. Percentages of DNA fragmentation were determined by counting radioactivity present in fragmented and intact DNA. The data represent the means $\pm$ S.D. for triplicate assays in a single experiment. The experiment was repeated three times with similar results. (**b**) The effects of PTX on LPA-mediated proliferation and survival from three independent experiments were averaged and are presented as percentage activity (means $\pm$ S.D.), with the activity of LPA in the absence of PTX defined as 100%.

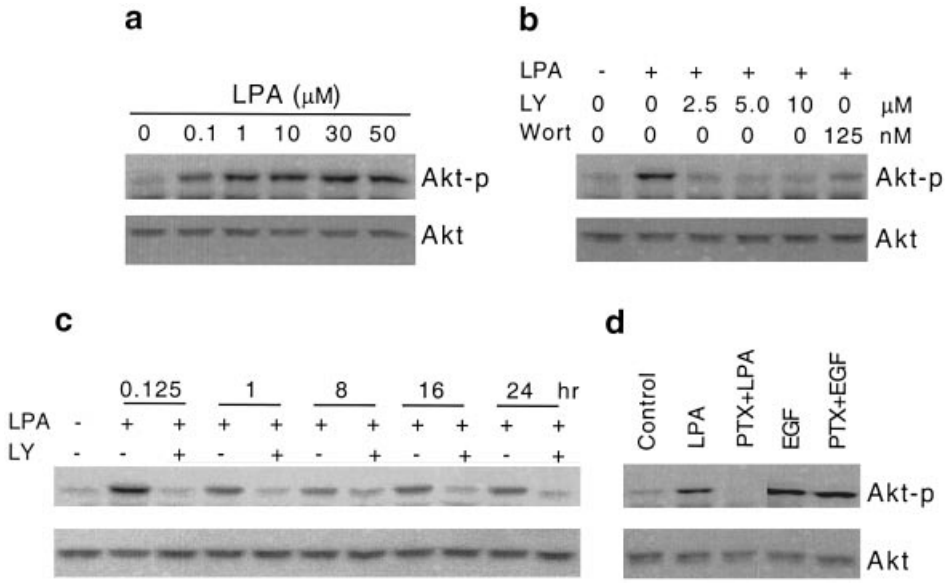

Figure 3 LPA stimulates PTX-sensitive phosphorylation/activation of Akt via PI3K

(**a**) NIH 3T3 cells were stimulated with the indicated concentrations of LPA for 7.5 min. Cell lysates were analysed by immunoblotting with an Akt phospho-specific antibody that recognizes Akt phosphorylated at Ser-473. The membrane was stripped and reprobed with an antibody raised against total Akt. (**b**) NIH 3T3 cells were stimulated with LPA (30 μM) in the absence or presence of the indicated concentrations of LY 294002 (LY) or wortmannin (Wort). LY or Wort was added 1 h before stimulation with LPA. (**c**) NIH 3T3 cells were stimulated with LPA (30 μM) with or without LY (10 μM), as in (**b**). The stimulation was terminated at the indicated time points up to 24 h (hr). (**d**) NIH 3T3 cells were stimulated for 7.5 min with LPA (30 μM) or EGF (25 ng/ml) alone or with PTX (25 ng/ml). The cells were incubated with PTX for at least 5 h before stimulation with LPA or EGF. For (**b**), (**c**) and (**d**), Akt phosphorylation was assayed by immunoblotting, as in (**a**).

mentation with LPA in serum-free medium efficiently blocked the apoptotic process, as shown by inhibition of morphological changes associated with apoptosis (Figure 1a) or by prevention of cellular DNA fragmentation (Figure 1b). As shown in NIH 3T3 cells, the effects of LPA on cell survival and proliferation were dependent on LPA concentrations with suboptimal stimulation (75–85%) at 30 μM LPA (Figures 1b and 1c). In Rat-1 cells, an effect of LPA could not be readily assessed, since the cells were relatively resistant to serum deprivation, and incubation for up to 24 h in the absence of serum resulted in few apoptotic cells (results not shown). We then utilized the Rat1/c-Myc cell line, which expresses constitutively the human c-*myc* proto-oncogene. The cell line undergoes robust apoptosis upon serum withdrawal [23]. A similar survival effect of LPA was observed in Rat1/c-Myc cells (Figure 1a and 1b). The anti-apoptotic role of LPA was also manifest in other non-fibroblast cell lines, such as Jurkat and HeLa cells (results not shown), indicating that the activity is not restricted to cells of fibroblast origin.

The major survival activity of LPA is mediated by a PTX-sensitive pathway

LPA stimulates its biological responses via specific G-protein-coupled receptors [1]. A variety of G-proteins are implicated in the initiation of the pleiomorphic cellular responses to LPA [1,4]. For instance, LPA-stimulated cell proliferation is dependent on a G_i-protein-mediated signalling network, as evidenced by inhibition of the mitogenic activity of LPA by PTX [4]. To address the role of G_i proteins in LPA-mediated prevention of apoptosis, we compared the impact of PTX on LPA-induced proliferation and survival. The experiments described below will focus on 3T3 cells, as these exhibit normal cell-cycle regulation and are readily synchronized at the G_0–G_1 phase of the cell cycle following brief starvation, whereas it is hard to enforce quiescence in Rat1/c-Myc cells because of the constitutive expression of c-Myc [23]. Therefore, in 3T3 cells, the effects of pharmaceutical inhibitors on cell proliferation and apoptosis can be assessed in parallel. To inactivate G_i proteins, NIH 3T3 cells were preincubated with PTX (25 ng/ml) at least 5 h before addition of LPA. PTX inhibited LPA's proliferative activity by more than 90%, as assessed by [^{3}H]thymidine incorporation (Figure 2). It also caused an average of 75% inhibition of the anti-apoptotic activity of LPA, resulting in a dramatic increase in DNA fragmentation (Figure 2). As a control, PTX did not affect the survival activity of PDGF, and only slightly decreased the proliferative effect of PDGF (10%), an agonist that does not involve a G_i protein in its signal transduction. These results suggest a specific role for PTX-sensitive G_i proteins in LPA-mediated cell proliferation and survival, although the possibility cannot be ruled out that other minor G_i-protein-independent pathway(s) may also contribute to the survival activity of LPA.

LPA stimulates phosphoinositide 3-kinase (PI3K)-dependent activation of Akt in a PTX-sensitive manner

Previous results employing mutant receptors of insulin-like-growth-factor-1 and inhibitors of PI3K suggest that PI3K transduces survival signals [29]. Later work identified the serine/threonine kinase, Akt/PKB, as a downstream target of PI3K, which, in turn, phosphorylates/inactivates pro-apoptotic proteins BAD (**B**cl-2/Bcl-X_L-**a**ntagonist, causing cell **d**eath) [30], caspase 9 [31] and FKHRL1 [32], thereby promoting cell survival. We therefore examined the potential involvement of the PI3K–Akt pathway in cell survival elicited by LPA. Although previous studies failed to show an ability of LPA to activate Akt in fibroblasts and PC12 cells [33,34], we have detected a modest but

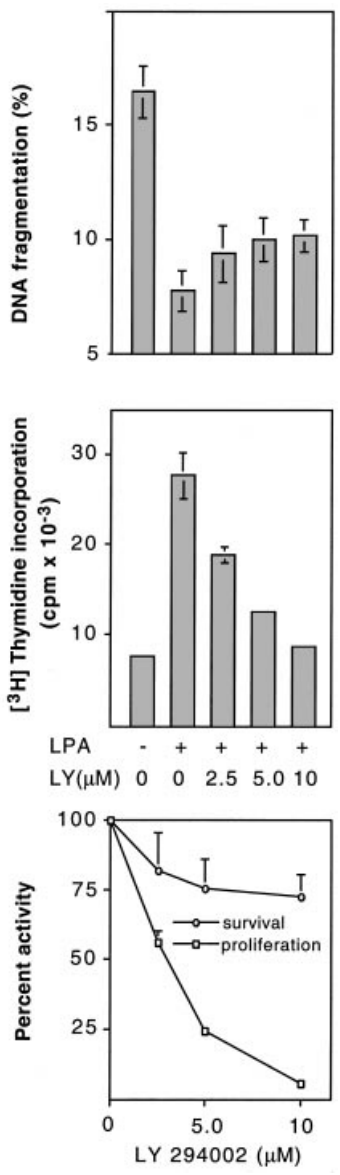

Figure 4 LPA-mediated proliferation and survival demonstrate differential dependence on PI3K activity

NIH 3T3 cells were incubated with LPA (30 μM) in the presence of the indicated concentrations of LY 294002. Cell proliferation was measured by [^{3}H]thymidine incorporation, as described in the Experimental section. Cell survival was quantified in parallel by measuring cellular DNA fragmentation. [^{3}H]Thymidine-pre-labelled cells were incubated in serum-free medium with vehicle or LPA for 16–18 h before harvesting. Percentages of DNA fragmentation were determined by counting radioactivity present in fragmented and intact DNA. For both cell proliferation and survival, LY 294002 was added 1 h before LPA. In the bottom panel, the effects of LY 294002 on cell proliferation and survival were plotted as percentage activity, with the activity of LPA in the absence of the inhibitor defined as 100%. The data represent an experiment of triplicate assays (means $\pm$ S.D.). Similar results were obtained from three independent experiments.

consistent level of Akt phosphorylation in response to LPA in NIH 3T3 and Swiss 3T3 cells by using a phosphorylation/activation-specific antibody that recognizes Akt phosphorylated at Ser-473 (Figure 3a). LPA also stimulated Akt phosphorylation in other cell lines tested, including OVCAR-3 (human ovarian carcinoma cell line), and Rat-1 (X. Fang and G. B. Mills, unpublished work). A modest increase in Akt kinase activity following LPA stimulation of 3T3 cells was also detected by an *in vitro* kinase assay using histone 2B as a substrate (results not shown). Akt phosphorylation in response to LPA was dependent on PI3K activity, because the phosphorylation was abolished by the PI3K inhibitors LY 294002 and wortmannin (Figure 3b), indicating that LPA stimulates Akt phosphorylation/activation via PI3K. The inhibition of Akt phosphorylation by LY 294002 was persistent for up to 24 h of incubation (Figure 3c)

To determine whether LPA-induced activation of the PI3K–Akt pathway is mediated by a G_i protein, Akt phosphorylation by LPA was examined in NIH 3T3 cells pretreated with or without PTX. At the same concentration that effectively blocked LPA's survival and proliferative activity (see Figure 2), PTX completely inhibited LPA-induced Akt phosphorylation, without compromising that stimulated by EGF (Figure 3d) or PDGF (results not shown). Thus a PTX-sensitive G_i protein is involved in LPA activation of the PI3K–Akt cascade.

LPA-induced proliferation and survival demonstrate differential requirements for PI3K activity

PTX-sensitive activation of the PI3K–Akt cascade by LPA highlights a potential role of this network in transduction of LPA's survival signal. We therefore examined the effects on LPA-induced cell proliferation and survival of LY 294002 and wortmannin, two specific inhibitors that exert their effects on PI3K via distinct mechanisms [29–32]. In NIH 3T3 cells, LY 294002 inhibited LPA-stimulated DNA synthesis in a dosage-dependent manner. As shown in Figure 4, treatment of cells with 2.5, 5.0 and 10 μM of LY 294002 led to 44, 76 and 95% inhibition respectively of [^{3}H]thymidine incorporation stimulated by LPA. A weaker but similar effect was seen with wortmannin (results not shown). These results demonstrate that PI3K activity is required for LPA-induced cell proliferation, consistent with a general role for PI3K in cell proliferation.

In parallel experiments using the same batches of cells, however, the protection of cells from apoptosis due to LPA was inhibited by LY 294002 or wortmannin to a much lesser extent than was the proliferative effect of LPA (Figure 4). For instance, in the presence of LY 294002 at 2.5, 5.0 and 10 μM, LPA-mediated prevention of DNA fragmentation was only inhibited by 18, 25 and 28% respectively. With wortmannin, the inhibition was even weaker (10–20%), in spite of a >60% decrease in DNA synthesis (results not shown). The differential effects of PI3K inhibitors on proliferation and survival were consistent with different batches of LY 294002 and wortmannin in multiple independent experiments in NIH 3T3 and Swiss 3T3 lines. The results suggest that only a minor fraction of LPA's survival activity is contributed by the PI3K pathway.

LPA activates MAPK/ERK independently of PI3K

The experiments described above suggest a minor role for the PI3K pathway in LPA-conferred cell survival. We then considered whether LPA stimulates survival via activation of MAPK/ERK, as this pathway represents another major survival regulator. In particular, ERK activity is required for cell survival mediated by sphingosine 1-phosphate, a bioactive phospholipid that is structurally similar to LPA [28]. As described previously [1], LPA induced potent activation of ERK1 and ERK2, as reflected by gel-mobility shift assay or by an increase in levels of phosphorylated ERK1/ERK2 recognized by an ERK phospho-specific antibody (Figure 5a). LPA activation of ERK was sensitive to the action of PTX (Figure 5a, right panel).

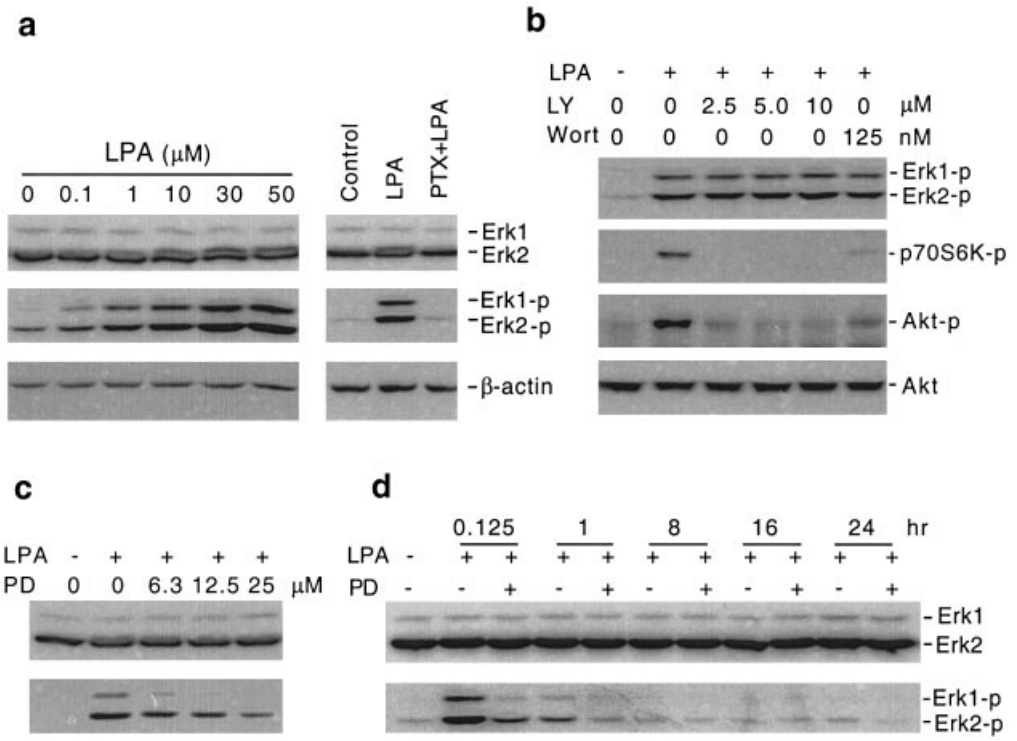

Figure 5 LPA activates MAPK/ERK independently of PI3K

(**a**) LPA induces PTX-sensitive activation/phosphorylation of ERK. NIH 3T3 cells were stimulated (7.5 min) with various concentrations of LPA as indicated (left panel), or with a fixed concentration of LPA (30 μM) in the absence or presence of PTX (25 ng/ml) (right panel). PTX was added to the culture at least 5 h before LPA stimulation. Cell lysates were analysed by immunoblotting for ERK2 mobility shift using an anti-ERK2 antibody, which weakly cross-reacted with ERK1. Phosphorylation of ERK was also analysed by immunoblotting using an ERK1/ERK2 phospho-specific antibody that recognized ERK1/ERK2 phosphorylated at Thr-183 and Tyr-185. Bands corresponding to ERK1 and ERK2 or phosphorylated ERK1 (Erk1-p) and ERK2 (Erk2-p) are indicated on the right-hand side of the right panels. (**b**) LPA-induced ERK phosphorylation is resistant to PI3K inhibitors. NIH 3T3 cells were stimulated with LPA (30 μM, 7.5 min) in the presence of the indicated concentrations of LY 294002 (LY) or wortmannin (Wort). Phosphorylation of ERK1/ERK2, p70 S6K (Thr-389) and Akt (Ser-473) was analysed by immunoblotting using phospho-specific antibodies. Total Akt was included to show equal protein loading. (**c**) and (**d**) LPA-induced ERK phosphorylation is sensitive to the MEK-1 inhibitor PD 98059 (PD). In (**c**), NIH 3T3 cells were stimulated with LPA (30 μM, 7.5 min) in the presence of the indicated concentrations of PD. In (**d**), NIH 3T3 cells were incubated with LPA (30 μM) with or without PD (25 μM) for the indicated intervals. Phosphorylation of ERK1 and ERK2 by LPA was assayed by immunoblotting as in (**a**). Phosphorylated forms of ERK1, ERK2, p70 S6K and Akt are shown in (**a**), (**b**) and (**d**) by Erk1-p, Erk2-p, p70 S6K-p and Akt-p.

Several previous studies suggest a modulatory role for PI3K activity in stimulation of ERK by various ligands, including LPA [35]. However, in NIH 3T3 or Swiss 3T3 cells, we did not observe any detectable effect of the PI3K inhibitors, LY 294002 or wortmannin, on LPA-stimulated ERK phosphorylation/activation (Figure 5b). In contrast, the same concentrations of these inhibitors completely prevented LPA-induced phosphorylation/activation of other kinases known to be downstream of PI3K, such as Akt and p70 S6 kinase (Figure 5b), suggesting that ERK1/ERK2 activation by LPA is independent of functional PI3K, at least in NIH 3T3 and Swiss 3T3 cells with the LPA concentrations tested.

MAPK/ERK activation is required for both proliferation and survival stimulated by LPA

To assess the role of the MAPK/ERK pathway in LPA-mediated cell proliferation and survival, NIH 3T3 cells were incubated with LPA in the presence of different concentrations of PD 98059, a MEK-1 inhibitor that has been shown to specifically block growth-factor-induced ERK activation in a wide range of cell lines [36]. As shown in Figures 5(c) and 5(d), PD 98059 inhibited LPA-stimulated activation of ERK1/ERK2 in NIH 3T3 cells. Consistent with an essential role for ERK in the induction of cell proliferation, treatment with PD 98059 caused a dose-dependent inhibition of LPA-induced DNA synthesis (Figure 6). At a concentration of PD 98059 of 25 μM, LPA-stimulated DNA synthesis was inhibited by more than 90%. Differently from LY 294002 or wortmannin, however, PD 98059 also suppressed LPA's survival activity by approx. 70%. Similar results were obtained with Swiss 3T3 cells treated with PD 98059. These data, combined with the observation that PTX also suppressed the anti-apoptotic effect of LPA, suggest that the majority of LPA's survival activity is dependent on G_i-protein-mediated activation of the classical MAPK/ERK pathway. As it has been reported that PD 98059 also inhibits activation of other MAPK family members, such as ERK5 [37], the possibility cannot be ruled out that ERK5 could be mediating or contributing to the cell-survival effect of LPA.

DISCUSSION

Although LPA-stimulated cell proliferation has been extensively studied [1,4], LPA as a regulator of cell survival is a relatively new concept lacking vigorous investigation. In the present study, we have demonstrated that LPA is a potent survival factor inhibiting serum-withdrawal-induced apoptosis in fibroblasts and other cell types. Similar to the LPA-induced proliferative

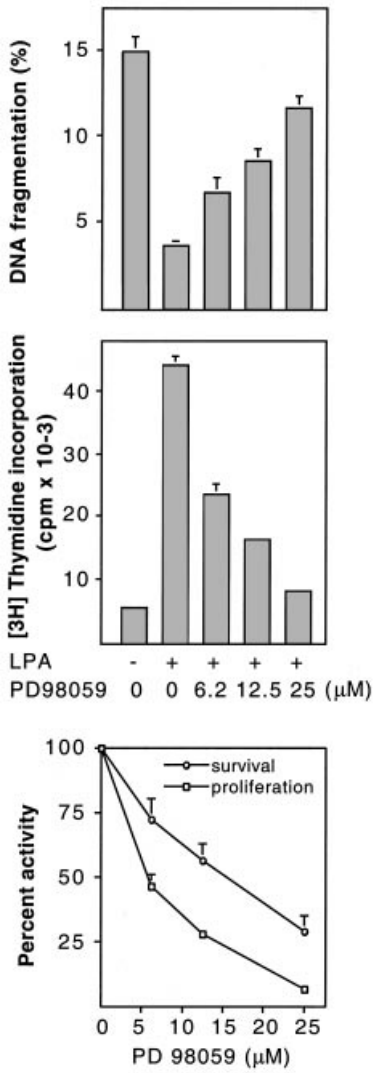

Figure 6 ERK activation is required for both proliferation and survival stimulated by LPA

NIH 3T3 cells were incubated with LPA (30 μM) in the presence of the indicated concentrations of PD 98059. Cell proliferation and survival were assessed in parallel, as described in the legend to Figure 4. In the bottom panel, the effects of PD 98059 on LPA-mediated cell proliferation and survival were plotted as percentage activity against PD 98059 concentration, with that of LPA in the absence of the inhibitor defined as 100%. The data represent an experiment of triplicate assays (means $\pm$ S.D.). Three independent experiments were performed, yielding similar results.

response, LPA-mediated cell survival, or at least the majority of the activity, is dependent on a PTX-sensitive G_i protein(s), revealing an overlapping relay required for both responses to LPA. In addition to activating MAPK/ERK via a G_i protein as described previously [1,4], LPA also stimulates PTX-sensitive activation of the PI3K–Akt pathway. When specific pharmaceutical inhibitors were used to assess the roles of these G_i-protein-mediated signaling events, we found that both pathways are essential for the mitogenic activity of LPA, but the MAPK/ERK pathway plays a more critical role than the PI3K–Akt module in the promotion of cell survival by LPA.

The activity of MAPK/ERK is essential for cell proliferation and differentiation. In recent years, it has become increasingly evident that members of the MAPK family play important roles in the control of cell life and death. While stress-activated MAPK members [Janus kinase/stress-activated protein kinase ('JNK/SAPK')] and p38 HOG kinases function as death mediators during apoptosis induced by UV light or other types of stress, ERK exhibits opposing effects, promoting cell survival under many different conditions [38,39]. ERK activity has been shown to be critical to cell survival mediated by multiple diverse growth/survival factors [28,40,41]. In other cases, MAPK/ERK activity suppresses or desensitizes apoptosis triggered by a wide range of death stimuli, such as Fas ligand, radiation and chemotherapeutic agents [42–44]. Although mechanisms for the anti-apoptotic action of MAPK/ERK have not been well characterized, it appears that multiple effectors are involved. More recent studies demonstrate that some death/survival mediators, such as BAD and CREB ('cAMP-response-element-binding protein'), previously considered to be effectors of other survival pathways, can also be inactivated or activated by Rsk, a kinase downstream of ERK [39,45,46].

PI3K is believed to exert its anti-apoptotic function via activation of Akt, which subsequently phosphorylates/inactivates BAD and other pro-apoptotic proteins, such as caspase 9 and the forkhead transcription factor, FKHRL1 [30–32]. PI3K thus links proximal survival factors to inactivation of a number of critical components of the cell-intrinsic death machinery. Similarly to other survival factors, LPA stimulates PI3K-dependent activation of Akt. However, inhibition of PI3K activity by treatment with LY 294002 or wortmannin blocked only a limited fraction of the anti-apoptotic activity of LPA. This is not due to insufficient dosages of the inhibitors, because at the same range of concentrations these inhibitors very efficiently blocked the mitogenic activity of LPA and completely eliminated LPA-stimulated phosphorylation/activation of Akt and p70 S6 kinase. Therefore, compared with ERK, PI3K does not appear to be a primary mediator of LPA-induced cell survival in fibroblasts. This conclusion is compatible with the observation that the ability of LPA to induce PI3K-dependent activation of Akt was modest, and was not even detected in some previous reports [33,34]. Our results support a number of recent studies in which death suppression can be accomplished without activation of Akt or through PI3K-independent mechanisms [47–49]. Therefore the role of PI3K in the cell survival decision depends on the cell type, the apoptotic model or the signal strength of the pathway.

Our results are not in accordance with studies by Levine and co-workers, who recently described that LPA's prevention of apoptosis in renal proximal tubular cells [17] and murine macrophages [18] was dependent on PI3K activity. It is possible that the role of the PI3K pathway in LPA-induced cell survival may vary among cell types. The discrepancy could also be related to the different ways in which the effect of PI3K inhibitors was assessed. In their experiments, the effect of wortmannin and LY 294002 on LPA-mediated cell survival was determined after a 3–10 day period of continuous incubation, different from the short-term approach used in the present study. In addition to PI3K, wortmannin and LY 294002 also inhibit class IV PI3K-related kinases, including Atm, Atr, rapamycin and FK506-binding protein ('RAFT'), DNA-dependent protein kinase ('DNA-PK'), as well as unrelated enzymes, such as phospholipase A_2 [50]. Long-term, persistent inhibition of these other enzymes may have non-specific effects on cell viability.

Our results also suggest a fundamental difference between the proliferative and survival signals activated by LPA. LPA-mediated cell proliferation apparently requires activation of multiple signal-transduction pathways. Inhibition of individual cascades, at the level of MAPK/ERK, PI3K or p70 S6 kinase,

resulted in a substantial block of DNA synthesis in response to LPA, suggesting that integration or co-operation of signals from multiple pathways is necessary to drive cells to enter S-phase. However, the survival activity of LPA does not seem to require signal integration from multiple pathways. Thus the survival function of LPA can be dissociated from, and is independent of, its mitogenic activity. For example, as shown in Figure 4, LPA remains capable of supporting cell survival when its commitment to cell proliferation is abrogated by LY 294002.

Demonstration of LPA's anti-apoptotic effect suggests that LPA may contribute to the survival activity of serum. Serum has long been known to contain survival-promoting activity, which has been mainly attributed to PDGF and insulin-like growth factor. Identification of LPA as an anti-apoptotic factor provides a novel insight into the potential function *in vivo* of this biomediator. It has been suggested that LPA is rapidly and locally produced in response to injury and other stress conditions [1,2]. Under these circumstances, LPA might play a dual role: protection of cells from undergoing apoptosis, and stimulation of cell division to restore tissue mass. Interestingly, sphingosine 1-phosphate has been also shown to be an inhibitor of apoptosis [28], making it likely that these non-peptide phospholipids represent a novel family of survival factors.

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REFERENCES

1 Moolenaar, W. H. (1995) Lysophosphatidic acid, a multifunctional phospholipid messenger. J. Biol. Chem. **270**, 12949–12952
2 Eichholtz, T., Jalink, K., Fahrenfort, I. and Moolenaar, W. H. (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. Biochem. J. **291**, 677–680
3 Gaits, F., Fourcade, O., Le Balle, F., Gueguen, G., Gaige, B., Gassama-Diagne, A., Fauvel, J., Salles, J. P., Mauco, G., Siman, M. F. and Chap, H. (1997) Lysophosphatidic acid as a phospholipid mediator: pathways of synthesis. FEBS. Lett. **410**, 54–58
4 van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T. and Moolenaar, W. H. (1989) Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. Cell. **59**, 45–54
5 Gerrard, J. M., Kindom, S.E., Peterson, D. A., Peller, J., Krantz, K. E. and White, J. G. (1979) Lysophosphatidic acids. Interaction of the effects of adenosine diphosphate and lysophosphatidic acids in dog, rabbit, and human platelets. Am. J. Pathol. **96**, 423–438
6 Toews, M. L., Ustinova, E. E. and Schultz, H. D. (1997) Lysophosphatidic acid enhances contractility of isolated airway smooth muscle. J. Appl. Physiol. **83**, 1216–1222
7 Imamura, F., Horai, T., Mukai, M., Shinkai, K., Sawada, M. and Akedo, H. (1993) Induction of in vitro tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D. Biochem. Biophys. Res. Commun. **193**, 497–503
8 Ridley, A. J. and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell **70**, 389–399
9 Xu, Y., Gaudette, D. C., Boynton, J. D., Frankel, A., Fang, X. J., Sharma, A., Hurteau, J., Casey, G., Goodbody, A., Mellors, A., Holub, B. J. and Mills, G. B. (1995) Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients. Clin. Cancer Res. **1**, 1223–1232
10 Xu, Y., Shen, Z., Wiper, D. W., Wu, M., Morton, R. E., Elson, P., Kennedy, A. W., Belinson, J., Markman, M. and Casey, G. (1998) Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. JAMA, J. Am. Med. Assoc. **280**, 719–723
11 Goetzl, E. J. and An, S. (1999) A subfamily of G protein-coupled cellular receptors for lysophospholipids and lysosphingolipids. Adv. Exp. Med. Biol. **469**, 259–264
12 van der Bend, R. L., de Widt, J., van Corven, E. J., Moolenaar, W. H. and van Blitterswijk, W. J. (1992) The biologically active phospholipid, lysophosphatidic acid, induces phosphatidylcholine breakdown in fibroblasts via activation of phospholipase D: comparison with the response to endothelin. Biochem. J. **285**, 235–240
13 Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J. and de Laat, S. W. (1986) Growth factor-like action of phosphatidic acid. Nature (London) **323**, 171–173
14 Howe, L. R. and Marshall, C. J. (1993) Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring p21ras and p74raf-1. J. Biol. Chem. **268**, 20717–20720
15 Seufferlein, T. and Rozengurt, E. (1994) Lysophosphatidic acid stimulates tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130. Signaling pathways and cross-talk with platelet-derived growth factor. J. Biol. Chem **269**, 9345–9351
16 Frankel, A. and Mills, G. B. (1996) Peptide and lipid growth factors decrease *cis*-diamminedichloroplatinum-induced cell death in human ovarian cancer cells. Clin. Cancer Res **2**, 1307–1313
17 Levine, J. S., Koh, J. S., Triaca, V. and Lieberthal, W. (1997) Lysophosphatidic acid: a novel growth and survival factor for renal proximal tubular cells. Am. J. Physiol. **273**, F575–F585
18 Koh, J. S., Lieberthal, W., Heydrick, S. and Levine, J. S. (1998) Lysophosphatidic acid is a major serum noncytokine survival factor for murine macrophages which acts via the phosphatidylinositol 3-kinase signaling pathway. J. Clin. Invest **102**, 716–727
19 Goetzl, E. J., Kong, Y. and Mei, B. (1999) Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. J. Immunol **162**, 2049–2056
20 Weiner, J. A. and Chun, J. (1999) Schwann cell survival mediated by the signaling phospholipid lysophosphatidic acid. Proc. Natl. Acad. Sci. U.S.A. **96**, 5233–5238
21 Holtsberg, F. W., Steiner, M. R., Keller, J. N., Mark, R. J., Mattson, M. P. and Steiner, S. M. (1998) Lysophosphatidic acid induces necrosis and apoptosis in hippocampal neurons. J. Neurochem. **70**, 66–76
22 Holtsberg, F. W., Steiner, M. R., Bruce-Keller, A. J., Keller, J. N., Mattson, M. P., Moyers, J. C. and Steiner, S. M. (1998) Lysophosphatidic acid and apoptosis of nerve growth factor-differentiated PC12 cells. J. Neurosci Res **53**, 685–696
23 Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z. and Hancock, D. C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. Cell **69**, 119–128
24 Duke, R. C., Cohen, J. J., Boehme, S. A., Lenardo, M. J., Surh, C. D., Kishimoto, H. and Sprent, J. (1995) Morphological, biochemical and flow cytometric assays of apoptosis. In Current Protocols in Immunology (Coligan, J. E, Kruisbeek, A. M, Marguiies, D. H, Shevach, E. M and Strober, W., eds.), pp. 3.17.4.–3.17.7, John Wiley & Sons, Inc., New York
25 Franke, T. F., Yang, S., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. and Tsichlis, P. N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell **81**, 727–736
26 Chou, C. C. and Yung, B. Y. (1997) Antiapoptotic effect of ras in the apoptosis induced by serum deprivation and exposure to actinomycin D. Naunyn Schmiedebergs Arch. Pharmacol **355**, 177–182
27 Goruppi, S., Ruaro, E. and Schneider, C. (1996) Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts. Oncogene **12**, 471–480
28 Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. M., Gutkind, J. S. and Spiegel, S. (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature (London) **381**, 800–803
29 Kulik, G., Klippel, A. and Weber, M. J. (1997) Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol. Cell. Biol. **17**, 1595–1606
30 Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell **91**, 231–241
31 Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S. and Reed, J. C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. Science **282**, 1318–1321
32 Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J. and Greenberg, M. E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell **96**, 857–868
33 Burgering, B. M. and Coffer, P. J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature (London) **376**, 599–602
34 Andjelkovic, M., Suidan, H. S., Meier, R., Frech, M., Alessi, D. and Hemmings, B. A. (1998) Nerve growth factor promotes activation of the alpha, beta and gamma isoforms of protein kinase B in PC12 pheochromocytoma cells. Eur. J. Biochem **251**, 195–200
35 Kranenburg, O., Verlaan, I., Hordijk, P. L. and Moolenaar, W. H. (1997) Gi-mediated activation of the Ras/MAP kinase pathway involves a 100 kDa tyrosine-phosphorylated Grb2 SH3 binding protein, but not Src nor Shc. EMBO J **16**, 3097–3105
36 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. U.S.A. **92**, 7686–7689

37 Kamakura, S., Moriguchi, T. and Nishida, E. (1999) Activation of the protein kinase ERK5/BMK by receptor tyrosine kinases. J. Biol. Chem. **274**, 26563–26571
38 Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270**, 1326–1331
39 Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A. and Greenberg, M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and independent mechanisms. Science **286**, 1358–1362
40 Gupta, K., Kshirsagar, S., Li, W., Gui, L., Ramakrishnan, S., Gupta, P., Law, P. Y. and Hebbel, R. P. (1999) VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. Exp. Cell Res. **247**, 495–504
41 Hetman, M., Kanning, K., Cavanaugh, J. E. and Xia, Z. (1999) Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J. Biol. Chem **274**, 22569–22580
42 Holmstrom, T. H., Tran, S.E., Johnson, V. L., Ahn, N. G., Chow, S. C. and Eriksson, J. E. (1999) Inhibition of mitogen-activated kinase signaling sensitizes HeLa cells to Fas receptor-mediated apoptosis. Mol. Cell. Biol. **19**, 5991–6002
43 Persons, D. L., Yazlovitskaya, E. M., Cui, W. and Pelling, J. C. (1999) Cisplatin-induced activation of mitogen-activated protein kinases in ovarian carcinoma cells: inhibition of extracellular signal-regulated kinase activity increases sensitivity to cisplatin. Clin. Cancer Res **5**, 1007–1014
44 Dent, P., Reardon, D. B., Park, J. S., Bowers, G., Logsdon, C., Valerie, K. and Schmidt-Ullrich, R. (1999) Roles for basal and stimulated p21(Cip-1/WAF1/MDA6) expression and mitogen-activated protein kinase signaling in radiation-induced cell cycle checkpoint control in carcinoma cells. Mol. Biol. Cell **10**, 2493–2506
45 Fang, X., Yu, S., Eder, A., Mao, M., Bast, R. C., Boyd, D. and Mills, G. B. (1999) Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. Oncogene **18**, 6635–6640
46 Scheid, M. P., Schubert, K. M. and Duronio, V. (1999) Regulation of Bad phosphorylation and association with Bcl-x(L) by the MAPK/ERK kinase. J. Biol. Chem.. **274**, 31108–31113
47 Scheid, M. P. and Duronio, V. (1998) Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/Akt: involvement of MEK upstream of Bad phosphorylation. Proc. Natl. Acad. Sci. U.S.A. **95**, 7439–7444
48 Craddock, B. L., Orchiston, E. A., Hinton, H. J. and Welham, M. J. (1999) Dissociation of apoptosis from proliferation, protein kinase B activation, and BAD phosphorylation in interleukin-3-mediated phosphoinositide 3-kinase signaling. J. Biol. Chem. **274**, 10633–10640
49 Kulik, G. and Weber, M. J. (1998) Akt-dependent and -independent survival signaling pathways utilized by insulin-like growth factor I. Mol. Cell. Biol **18**, 6711–6718
50 Vanhaesebroeck, B. and Waterfield, M. D. (1999) Signaling by distinct classes of phosphoinositide 3-kinases. Exp. Cell Res. **253**, 239–254

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