The role of phosphatidylinositide-3-kinase in basal mitogen-activated protein kinase activity and cell survival

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Abstract Phosphatidylinositide-3-OH-kinase (PI 3-kinase) is an upstream activator of p42/p44 mitogen-activated protein kinase (MAPK), but the role of PI 3-kinase-dependent MAPK remains obscure. Here we demonstrate that in a variety of different cell types, PI 3-kinase inhibition results in an inhibition of MAPK in unstimulated cells but does not interfere with growth factor-, or TPA-induced MAPK activity. Furthermore, inhibition of either PI 3-kinase or MEK/MAPK results in cell death in serum-starved cells. We concluded that basal, but not induced MAPK activity is mediated by PI 3-kinase and that this PI 3-kinase-mediated MEK/MAPK activity is essential for cell survival in quiescent cells.

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Key words: PI 3-kinase; Constitutive MAPK activation; Wortmannin; LY294002; Cell survival

1. Introduction

The lipid and protein kinase phosphatidylinositide-3-OH-kinase (PI 3-kinase) synthesises phosphatidylinositol-3-phosphate, phosphatidylinositol-3,4-diphosphate, and phosphatidylinositol-3,4,5-triphosphate from phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-diphosphate respectively. Several PI 3-kinase isoforms, such as PI 3-kinase α, β and γ, have been described, which mostly consist of a catalytic and a regulatory subunit and, although catalysing similar biochemical reactions, seem to mediate specific biochemical functions (for a review see Vanhaesebroeck et al. [1]). The availability of the pharmacological PI 3-kinase inhibitors wortmannin [2] and LY294002 [3] as well as the availability of catalytically inactive dominant negative PI 3-kinase constructs has now led to discovery of a wide variety of biological processes involving PI 3-kinase, including inhibition of apoptosis, receptor-stimulated signalling, and the regulation of membrane traffic. In general, it is assumed that these effects are brought about by the binding of phosphatidylinositol-4,5-biphosphate and phosphatidylinositol-3,4,5-triphosphate to the pleckstrin homology domains of downstream effectors like the phosphatidylinositol-4,5-biphosphate/phosphatidylinositol-3,4,5-triphosphate-dependent kinase 1, which in turn is an upstream activator of protein kinase B (PKB, also known as c-Akt) [4]. Other important cellular targets of PI 3-kinase include BAD [5,6], p21Rac [7,8], S6 kinase (e.g. [4]), focal adhesion signalling [9,10], and the respiratory burst in granulocytes [11].

During the last years, several reports have described the effects of PI 3-kinase activity on activation of the mitogen-activated protein kinase (MAPK) pathway. Several investigators have found MAPK activation by insulin to be sensitive to the PI 3-kinase inhibitor wortmannin [12,13]. This phenomenon is, however, hypothesised to be cell type and stimulus specific [14]. The type of PI 3-kinase involved is subject to discussion as well; some reports claim PI 3-kinase α to be the activator of the MAPK pathway [15,16], whereas other publications suggest that PI 3-kinase γ [17] might exert this effect. Very recently, Bondeva et al. [18] demonstrated an increase in phosphorylated p42/p44 MAPK levels in serum-starved COS7 cells transfected with different PI 3-kinase γ hybrids. This effect was not dependent on lipid kinase activity or membrane localisation of PI 3-kinase γ, indicating that the physical interaction between PI 3-kinase and Ras [19] is not important for this effect. The importance, however, of PI 3-kinase for MAPK enzymatic activity remains relatively poor defined.

Cell survival is critically dependent on several intracellular signalling routes. As stated above, PI 3-kinase has been shown to play a pivotal role in cell survival by the subsequent activation of the protein kinase Akt/PKB and BAD. Both PKB and BAD, the last being a member of the Bcl-2 family, have important anti-apoptotic actions. Interestingly, BAD is not only a substrate for PKB but is also phosphorylated by the MAPK kinase MEK [20], linking the classical Ras-MAPK pathway to cell survival. This notion is supported by several studies, which show a role for this signalling module in cell survival [21,22]. The interplay of PI 3-kinase- and MAPK-associated signal transduction in cell survival remains unclear. Hence, we set out to investigate the role of basal PI 3-kinase activity in cellular MAPK activation. Furthermore we have investigated the influence of pharmacological inhibition of PI 3-kinase and MAPK on cell survival. Here we show that the PI 3-kinase inhibitors wortmannin and LY294002 are able to decrease constitutive MAPK activity but do not significantly effect epidermal growth factor (EGF)-, 12-0-tetradecanoylphorbol-13-acetate (TPA)-, or insulin-induced MAPK activation in A14 fibroblasts, murine macrophages, COS cells and Chinese hamster ovary (CHO) cells. In addition, in quiescent cells PI 3-kinase and activity of the MAPK pathway are essential for cell survival, suggesting that either MEK or MAPK

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is an important anti-apoptotic effector of PI 3-kinase under these conditions.

2. Materials and methods

2.1. Cell culture

A14 fibroblasts and COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS) in a humidified environment at 37°C and 5% CO_2. A murine spleen macrophage cell line (clone 4-4, [23]) and Chinese hamster ovary (CHO) cells were cultured in RPMI-1640 medium, with 10% FCS under the same conditions as described above. Cells were passaged every 2 days. Preincubations were done with final concentrations of 100 nM wortmannin, 2 μM forskolin, 20 μM PD098059 and 10 μM LY294002. All inhibitors were solved in DMSO and added to the media in a 1:1000 dilution. DMSO controls were without effect.

2.2. Kinase phosphorylation assays

Cells were grown on 8-cm diameter dishes and after extensive washing with PBS to remove all serum, the cells were serum starved for 24 h. Subsequently, the cells were preincubated for 10 min with the above mentioned inhibitors. Cells were stimulated for 15 min with 1 μg/ml insulin, 1 μg/ml TPA, 10 ng/ml EGF or 10% FCS as appropriate in the same medium. After stimulation, the cells were washed with ice cold PBS and lysed by scraping in 150 μl protein sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, 1 mg bromophenol blue). 50 μl of the lysates, corresponding with approximately 2×10^6 cells were loaded on a 10% SDS-PAGE gel and after protein separation the proteins were blotted onto PVDF membrane. To check for equal loading of protein in each lane, Coomassie Brilliant Blue staining of the PVDF membrane was performed. Subsequently, membranes were blocked in 2% bovine serum albumin in PBS, supplemented with 0.1% Tween-20 and washed three times for 10 min with 0.2% BSA in PBS supplemented with 0.1% Tween-20. Raf, MEK, MAPK and PKB activation were determined by incubating the immunoblots with antibodies against phosphorylated Raf (Ser^{259}), MEK ($Ser^{217/221}$), MAPK (Thr^{202}/Tyr^{204}) and PKB (Ser^{473}), used in a 1:1000 dilution for 1 h. The blots were washed three times with wash buffer for 10 min and subsequently secondary antibody incubations were performed for 1 h with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (GAR-PO) in a 1:2000 dilution. After enhanced chemoluminescence using Lumilight$^+$ substrate, antibody binding was visualised using a Lumi-imager.

2.3. Survival assay

A14 cells were plated in 24-well plates and cultured in serum-free DMEM or serum-containing DMEM. Before using serum-free DMEM, the cells were washed twice with 10 ml of phosphate-buffered saline (PBS) prior to adding serum-free DMEM, in order to remove all serum. The cells were left untreated or treated with either 20 μM PD98059 or 10 μM LY294002. Cell survival was measured for 5 subsequent days according to the method described by Rubinstein et al., 1990 [24]. Shortly, cells were incubated with 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which was added directly into the media in a 10× concentrated solution, for 30′ at 37°C. Subsequently, the cells were lysed in isopropanol/0.04 N HCl. The solution was transferred to a 96-well plate and the $OD_{590/650}$ was determined. Survival was determined as a percentage of untreated cells.

The stability of PD95059 in serum-containing medium was determined by adding the compound to the cells in a final concentration of 20 μM. Cells were lysed after 2, 4, 8, 24 and 48 h. Samples were compensated for the total amount of protein, and loaded on a 10% SDS-PAGE, after which Western blotting was performed. Detection of MAPK phosphorylation was performed as described above.

2.4. Materials

DMEM and FCS were from Gibco and RPMI-1640 was from Bio-Whittaker. PD98059, forskolin, EGF, insulin, TPA, wortmannin, BSA and MTT were purchased from Sigma. LY294002 was from Alexis/Kordia. All phosphospecific antibodies were obtained from New England Biolabs, secondary antibody (GAR-PO) was obtained from DAKO. Lumilight$^+$ substrate and the Lumi-imager were from Boehringer Mannheim.

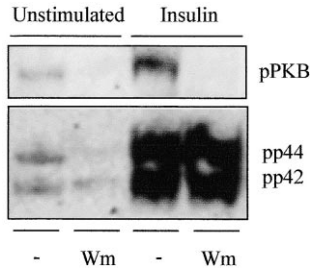


Fig. 1. Wortmannin inhibits basal, but not insulin-induced MAPK phosphorylation. After serum starvation, A14 cells were incubated with insulin for 15 min or left untreated, either in the presence or the absence of wortmannin (Wm). After cell lysis, the proteins were separated on a 10% SDS-PAGE and subsequently immunoblotted with polyclonal phosphospecific p42/p44 or phosphospecific PKB antibodies.

3. Results

3.1. The PI 3-kinase inhibitor wortmannin inhibits basal, but not stimulated MAPK activation in fibroblasts

Both recent findings that transfection of cells with PI 3-kinase γ constructs resulted in MAPK activation [18] and the observation that wortmannin can inhibit MAPK activation in stimulated cells [12,13] raises problems as to the importance of endogenous PI 3-kinase in MAPK stimulation. Therefore, we decided to investigate the effect of endogenous PI 3-kinase on both stimulated and constitutive MAPK activity. To this end serum-starved A14 cells were pre-treated with the specific PI 3-kinase inhibitor wortmannin, and cells were subsequently stimulated with 100 ng/ml insulin or left unstimulated. After immunoblotting, the phosphorylation states of both MAPK and PKB were assayed by screening with phosphospecific antibodies. The assumption was made that increased immunoreactivity corresponded with activation of the signalling components involved [4,25]. As depicted in Fig. 1, untreated cells displayed constitutive phosphorylation of MAPK. A 10-min incubation of the cells with wortmannin, however, inhibited this constitutive MAPK phosphorylation. Furthermore, the wortmannin treatment completely abolished phospho-PKB immunoreactivity, demonstrating that inhibition of MAPK by wortmannin correlates with a concomitant inhibition of well-established downstream targets of PI 3-kinase. This was confirmed in [^{3}H]-phosphoinositide measurements in which the influence of wortmannin on PI 3-kinase activity was assessed directly (not shown). Stimulation of cells with insulin, however, did not result in a wortmannin-sensitive MAPK activation. Apparently, PI 3-kinase is required for basal MAPK activation in this cell type, but not for insulin-induced MAPK activation.

3.2. c-Raf and MEK are involved in the wortmannin-sensitive basal activation of MAPK

To obtain further information on this PI 3-kinase-dependent MAPK stimulation, A14 cells were treated with forskolin and PD98059, inhibitors of c-Raf [26] and MEK [27], respectively. Both treatments completely abolished constitutive MAPK phosphorylation, demonstrating that PI 3-kinase-dependent MAPK stimulation is mediated via the Raf/MEK pathway and does not involve an alternative signalling mechanism (Fig. 2A). Surprisingly, inhibition of MAPK by forskolin or PD098059 resulted in increased PKB phosphorylation

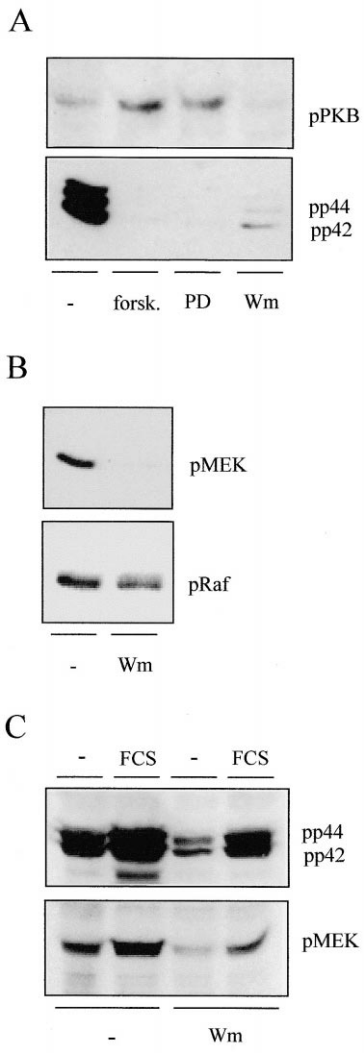


Fig. 2. MAPK activation in quiescent cells is mediated via c-Raf and MEK in a wortmannin-sensitive manner. A: After serum starvation and if appropriate, incubation with wortmannin (Wm), forskolin (forsk), PD098059 (PD), A14 cells were lysed, the proteins were separated on a 10% SDS-PAGE and immunoblotted with polyclonal phosphospecific p42/p44 (MAPK) or phosphospecific PKB antibodies. B: Cells were either or not treated with wortmannin for 15 min and lysates from these cells were used for immunoblotting with phosphospecific Raf and MEK antibodies. C: Serum-starved A14 fibroblasts either or not pre-incubated with wortmannin (Wm), were stimulated with 10% foetal calf serum (FCS) or left untreated. After cell lysis, proteins were separated on a 10% SDS-PAGE and immunoblotted with polyclonal phosphospecific antibodies.

(Fig. 2A). At present we have no data to explain the molecular basis of this effect but it is tempting to suggest that in unstimulated cells MAPK exerts negative feedback on PI 3-kinase activity, providing further support for an involvement of both c-Raf and MEK, since treatment of cells with wortmannin resulted in decreased c-Raf and MEK phosphorylation (Fig. 2B). In contrast to insulin-dependent MAPK stimulation, serum-induced MAPK and MEK activation was partly sensitive to wortmannin treatment (Fig. 2C), suggesting a role for PI 3-kinase in maintaining constitutive MAPK activity not only in quiescent cells, but in serum-treated cells as well.

3.3. Wortmannin and LY294002 inhibit basal MAPK activation in A14 cells, murine macrophages, COS cells and CHO cells

Apart from wortmannin, LY294002 is a specific inhibitor of PI 3-kinase activity. As expected, both inhibitors impaired constitutive MAPK activity in A14 fibroblasts (Fig. 3A). A14 cells overexpress the human insulin receptor, a strong activator of PI 3-kinase, and therefore regulation of this kinase in this cell type may not be representative. Therefore, we decided to investigate the role of PI 3-kinase in a panel of different cell types. As demonstrated in Fig. 3, in addition to A14 fibroblasts, PI 3-kinase inhibitors induce a decrease in basal MAPK phosphorylation in murine macrophages, COS cells and CHO cells as well, further confirming a role for PI 3-kinase in constitutive MAPK activation. Neither wortmannin nor LY294002, however, inhibited MAPK activation after stimulation with EGF or TPA in fibroblasts, macrophages and COS cells. CHO cells, in our hands, exhibited very high levels of basal MAPK phosphorylation, comparable to those observed after stimulation with various agents. However, whereas the basal levels of phosphorylated MAPK were again sensitive to both wortmannin and LY294002, insulin- and TPA-induced increases in phosphorylated MAPK were completely or partially insensitive to either wortmannin or LY294002 treatment. These data demonstrate that PI 3-kinase inhibitors do not abrogate signalling towards MAPK per se,

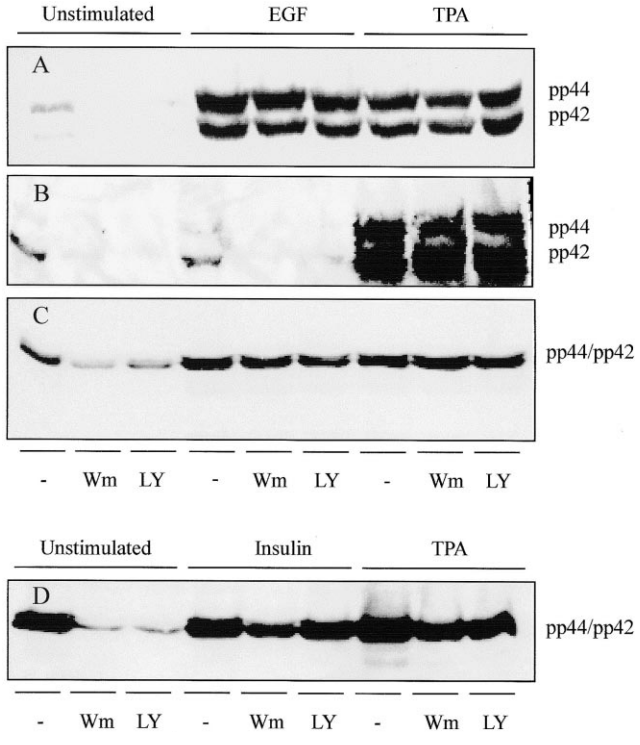


Fig. 3. Effects of LY94002 and wortmannin on constitutive and stimulated MAPK activation. After serum starvation, A14 cells (A), murine macrophages (B) and COS cells (C) were treated mock (unstimulated), with EGF, or with TPA, either in the presence or absence of wortmannin (Wm) and LY294002 (LY). CHO cells (panel D) were treated mock (unstimulated), with insulin or TPA, also in the presence or absence of wortmannin and LY294002. Subsequently, all cells were lysed, separated on 10% SDS-PAGE and immunoblotted with polyclonal phosphospecific p42/p44 antibodies.

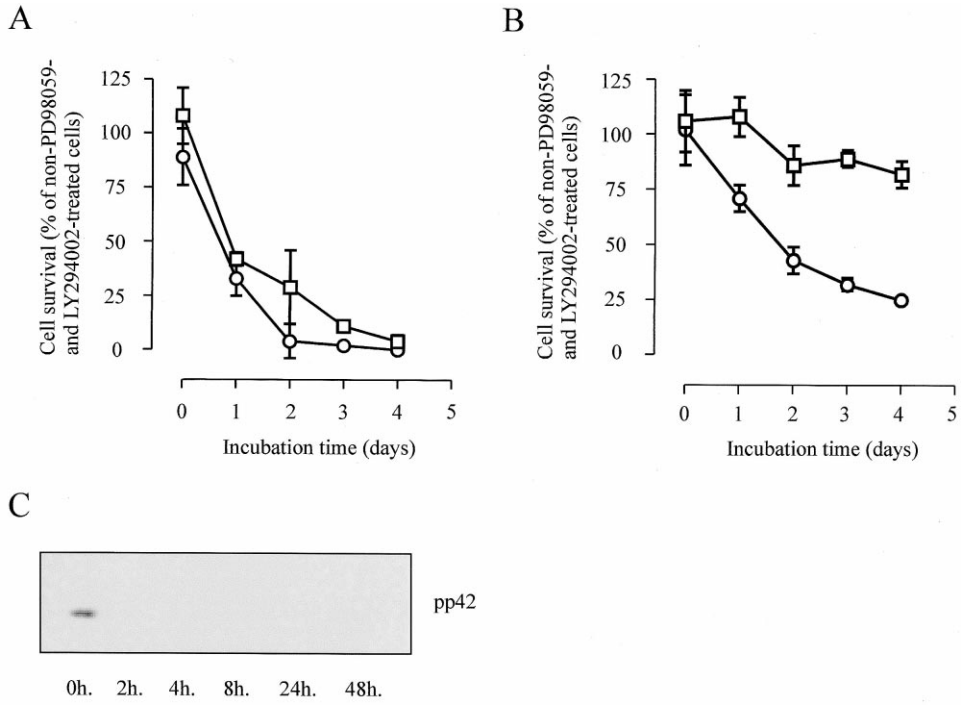


Fig. 4. Inhibition of MAPK in quiescent cells, but not in proliferating cells leads to cell death. A: A14 cells were plated and deprived of serum. After 1 day of serum starvation ($t=0$), cells were left untreated or subjected to either PD98059 or LY294002. Cell survival was determined for 4 days, by means of an MTT assay (see Section 2). B: Cells were plated and maintained in the presence of serum (FCS) and treated as described above. Lines indicated with dots represent cell survival after incubation with LY294002 for various times. Lines indicated with squares represent cell survival after incubation with PD98059 for various times. Experiments were performed in 8-fold, S.E.M. are depicted. C: Cells were incubated with PD98059 for various times and after lysis the samples were used for immunoblotting, using phosphospecific MAPK antibodies.

but that PI 3-kinase may therefore be specifically involved in the constitutive activation of MAPK.

3.4. Inhibition of basal MAPK and PI 3-kinase but not induced MAPK levels lead to cell death

The role of PI 3-kinase and its downstream targets in cell survival is well established. However, the action of the classical Ras-MAPK pathway in cell survival is much less defined. Therefore we decided to subject both serum-starved and serum-treated A14 cells to either the PI 3-kinase inhibitor LY294002 or to PD98059 by adding them to the cells prior to the experiment. In serum-starved cells, either of the two inhibitors proved sufficient to reduce cell survival, when compared to untreated cells (Fig. 4A). Serum-treated, proliferating cells also showed an LY294002-dependent increased cell death. However, treatment of serum-treated cells with PD98059 had little effect (Fig. 4B), and thus PD98059 is not toxic for cells per se. Addition of fresh PD98059 to the medium every day gave similar results (not shown), and when tested in serum-containing medium, PD98059 was able to inhibit MEK-dependent MAPK phosphorylation for at least 2 days (Fig. 4C), ruling out any effects caused by breakdown of PD98059 in the medium. Apparently, in quiescent cells both activation of the MAPK pathway and PI 3-kinase activity are required for cell survival, whereas in the presence of serum, activation of the MAPK pathway is not essential.

4. Discussion

Many intracellular processes responsible for cell survival have been unravelled in recent years. PI 3-kinase and its downstream targets PKB and BAD, play key roles in preventing cells from going into apoptosis. However, many aspects of the mechanisms behind cell survival have remained unclear. In this report we describe that basal PI 3-kinase is responsible for maintaining constitutive MAPK activation and that both wortmannin and LY294002 impair basal but not stimulated MAPK activity. Although studies performed with pharmacological agents are often inconclusive, both wortmannin and LY294002 are known to inhibit PI 3-kinase activity in different ways. Therefore, we conclude that the effects we observed are specific. Secondly, we demonstrate the requirement for both basal PI 3-kinase and basal MAPK activity in quiescent cells to prevent cell death to occur. Many reports have been made about wortmannin- and LY294002-sensitive MAPK activation by PI 3-kinase [12–14,28]. These studies, however, concerned the effects of PI 3-kinase inhibitors on insulin- or PDGF-stimulated MAPK activation, rather than basal activity of this kinase. We did not observe effects of wortmannin and LY294002 on MAPK activation after growth factor or phorbol ester stimulation. Wortmannin-sensitive MAPK activation by e.g. insulin may be cell type dependent. It is to be expected that such inhibition is only detected in cell types in which a weak coupling between growth factor receptors and the SOS-Ras pathway exists. In our study, we detected a role for PI 3-kinase in basal MAPK activation in four different cell types, therefore, in contrast to a role for PI 3-kinase in induced MAPK activity, its role in basal MAPK activity does not show much cell type specificity. Our report appears to be in contrast with the observations of e.g. Scheid and Duronio,

1996 [29] who demonstrate wortmannin to have no effect on MAPK activation in quiescent cells. Therefore it might be that PI 3-kinase-mediated MAPK activity in quiescent cells does not occur in all cells, but the data presented in our report definitely suggest that this may be a very common event. Our observations seem supported by recent findings. During our investigations, Wennstrom and Downward [16] have found a connection between PI 3-kinase α and MAPK in COS cells subjected to low dose EGF but not in cells subjected to high concentrations of EGF. This PI 3-kinase-mediated MAPK activation was mediated by Shc/Grb2/SOS complexes, and appears to account for EGF-induced MAPK stimulation at such concentrations that do not support recruitment of these complexes to the EGF receptor. From this observation these authors speculated that basal PI 3-kinase activity might be responsible for constitutive MAPK activity. Moreover, we observe that in variety of cell types constitutive MAPK phosphorylation is critically dependent on basal PI 3-kinase enzymatic activity. We have observed that the PI 3-kinase-induced constitutive MAPK stimulation is mediated via the Raf/MEK pathway, and it will be interesting to investigate whether Shc/Grb2/SOS complexes are involved in our experimental set up as well.

Interestingly, it has been suggested that integrin signalling is important in maintaining PI 3-kinase activity in quiescent cells [30,31], whereas the pivotal function of integrin signalling for cell survival in quiescent cells is also widely recognised [32,33]. Furthermore, also a function for integrin signalling in constitutive MAPK activity has been suggested [34]. In the present study we have demonstrated that both PI 3-kinase and one of the components of the MAPK pathway are required for cell survival of quiescent cells and that PI 3-kinase is the upstream activator of Raf, MEK and MAPK under these conditions. It is, therefore, tempting to suggest that integrin-mediated PI 3-kinase activation and subsequent stimulation of the MAPK pathway is the major pathway responsible for cell survival in quiescent cells. Although a role in cell survival has been attributed to MAPK [22], the effect witnessed in this investigation could also be ascribed to MEK since this MAPK kinase has also been reported to phosphorylate the anti-apoptotic protein BAD [5,6].

The nature of the PI 3-kinase isoform, responsible for the effects demonstrated in this report, remains unknown. Many authors have suggested the involvement of PI 3-kinase γ, since PI 3-kinase α constructs were not able to induce MAPK activity. However, the expression of PI 3-kinase γ seems limited to myeloid cells (e.g. [7,35]), suggesting involvement of another PI 3-kinase isoform. Wennstrom and Downward suggest a role for PI 3-kinase α in COS cells making it plausible that this isoform also regulates MAPK activity in our experimental set up. Experiments addressing this possibility are currently performed in our laboratory.

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