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PI 3-kinase γ and protein kinase C-ζ mediate RAS-independent activation of MAP kinase by a G_i protein-coupled receptor

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Receptors coupled to the inhibitory G protein G_i, such as that for lysophosphatidic acid (LPA), have been shown to activate MAP kinase through a RAS-dependent pathway. However, LPA (but not insulin) has now been shown to activate MAP kinase in a RAS-independent manner in CHO cells that overexpress a dominant-negative mutant of the guanine nucleotide exchange protein SOS (CHO-ΔSOS cells). LPA also induced the activation of MAP kinase kinase (MEK), but not that of RAF1, in CHO-ΔSOS cells. The RAS-independent activation of MAP kinase by LPA was blocked by inhibitors of phosphatidylinositol 3-kinase (PI3K) or by overexpression of a dominant-negative mutant of the γ isoform of PI3K. Furthermore, LPA induced the activation of the atypical ζ isoform of protein kinase C (PKC-ζ) in CHO-ΔSOS cells in a manner that was sensitive to wortmannin or to the dominant-negative mutant of PI3Kγ, and overexpression of a dominant-negative mutant of PKC-ζ inhibited LPA-induced activation of MAP kinase. These observations indicate that G_i protein-coupled receptors induce activation of MEK and MAP kinase through a RAS-independent pathway that involves PI3Kγ-dependent activation of atypical PKC-ζ.

Keywords: atypical PKC/G_i protein/LPA/MAP kinase/PI3Kγ

Introduction

The mechanism by which G protein-coupled receptors activate mitogen-activated protein (MAP) kinase has been studied extensively. In the case of receptors coupled to pertussis toxin (PTX)-sensitive G_i, such as those for lysophosphatidic acid (LPA), thrombin or α_2-adrenergic agonists, this pathway is initiated by the release of the βγ subunits from G_i (Crespo *et al*., 1994; Koch *et al*., 1994). Activation of G protein-coupled receptors results in the tyrosine phosphorylation of SH_2-containing protein (SHC), a reaction in which several tyrosine kinases have been implicated as playing a direct or indirect role (Touhara *et al*., 1995; van Biesen *et al*., 1996). Subsequent association of tyrosine-phosphorylated SHC with the adaptor protein GRB2 serves to direct the guanine nucleotide exchanger SOS to RAS. SRC family kinases have been suggested to mediate LPA- or Gβγ-induced tyrosine phosphorylation of SHC and subsequent activation of MAP kinase, given that overexpression of C-terminal SRC kinase (CSK), which inhibits the activity of SRC family kinases (Okada *et al*., 1991), markedly attenuated these responses (Dikic *et al*., 1996; Luttrell *et al*., 1996). LPA or bradykinin also induce activation of PYK2, which then forms a complex with and activates SRC in PC12 cells (Dikic *et al*., 1996). Furthermore, endothelin-1, LPA and thrombin each stimulate the tyrosine phosphorylation of epidermal growth factor receptors (a phenomenon known as transactivation), which is thought to result in the tyrosine phosphorylation of SHC and its association with GRB2 in Rat-1 fibroblasts (Daub *et al*., 1996).

Phosphatidylinositol 3-kinase (PI3K) has also been implicated in the activation of MAP kinase by G_i-coupled receptors. In COS-7 cells, LPA-induced activation of RAS and MAP kinase was blocked by inhibitors of PI3K, such as wortmannin or LY294002, or by a dominant-negative mutant of the p85 subunit of PI3K, suggesting that PI3K (presumably, the p110α or p110β isozyme) is required for G_i-mediated MAP kinase activation at a point upstream of RAS activation (Hawes *et al*., 1996). The PI3Kγ isozyme has also been suggested to mediate LPA- or Gβγ-induced tyrosine phosphorylation of SHC and activation of MAP kinase in COS-7 cells (Lopez-Ilasaca *et al*., 1997). However, it has been shown recently that neither SHC nor SRC is required for LPA-induced MAP kinase activation in Rat-1 fibroblasts (Kranenburg *et al*., 1997). These various studies indicate that the activation of RAS by various pathways, and the subsequent triggering of the RAF–MAP kinase kinase (MEK)–MAP kinase cascade, mediates MAP kinase activation by PTX-sensitive G_i (Howe and Marshall, 1993; Hordijk *et al*., 1994; Moolenaar, 1995).

We have shown previously that insulin fails to induce activation of RAS and MAP kinase in Chinese hamster ovary (CHO) cells that overexpress human insulin receptors and a dominant-negative mutant of murine SOS (CHO-ΔSOS cells) (Sakaue *et al*., 1995). In contrast, we have now shown that LPA induces PTX-sensitive activation of MAP kinase in CHO-ΔSOS cells to an extent similar to that observed in parental CHO cells overexpressing only human insulin receptors (CHO-IR cells). By analyzing these cells, we have identified a RAS-independent pathway for MAP kinase activation by G_i. This pathway involves an atypical isoform of protein kinase C (PKC), which may directly activate MEK, and the G_i-induced activation of atypical PKC appears to be mediated, at least in part, by PI3Kγ.

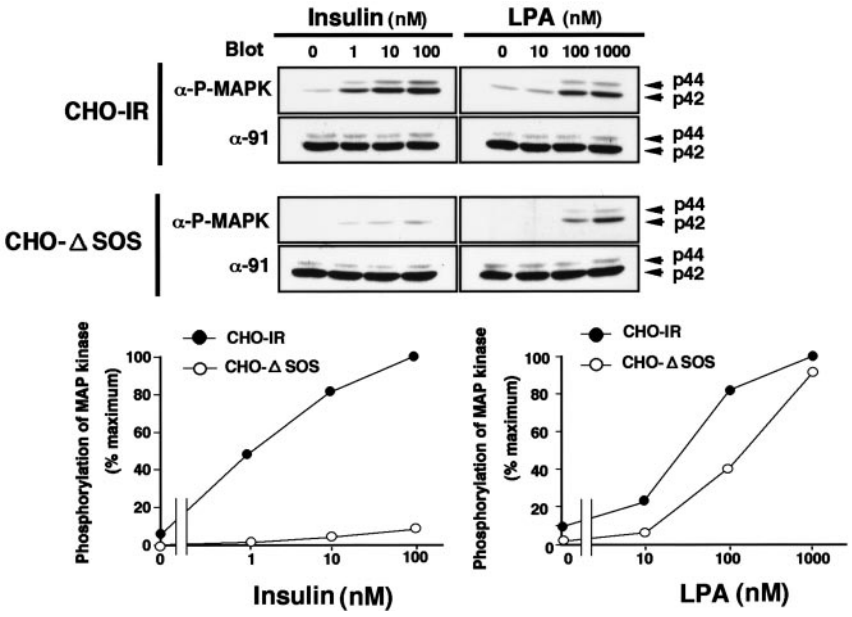


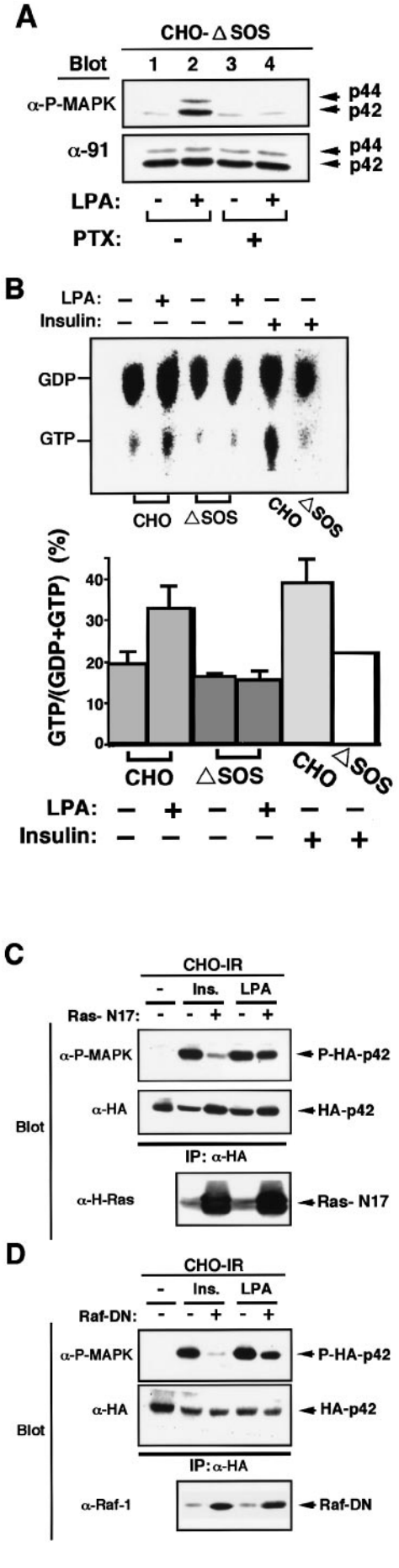
Fig. 1. Effects of LPA and insulin on MAP kinase activation in CHO-IR and CHO-ΔSOS cells. Cells were incubated for 5 min at 37°C with the indicated concentrations of insulin or LPA, after which the activated forms of p44 and p42 MAP kinase in whole-cell lysates were detected by immunoblot analysis with antibodies specific for the tyrosine-phosphorylated enzyme (α-P-MAPK) (upper panels). The same blot was also probed with α-91 polyclonal antibodies to p44 and p42 MAP kinase to confirm that equal amounts of enzyme were present in each lane (upper panels). The extent of tyrosine phosphorylation of p42 MAP kinase was quantified by scanning densitometry with the NIH image program (lower panels). Data are expressed as a percentage of the maximal value, apparent with 100 nM insulin or 1 μM LPA in parental CHO-IR cells, and are means of three independent experiments.

Results

Activation of MAP kinase in a RAS-independent manner by LPA (but not by insulin) in CHO-ΔSOS cells

We have shown previously that insulin fails to induce activation of RAS and MAP kinase in CHO-IR cells that overexpress a catalytically inactive mutant of the SRC homology 2 domain-containing protein tyrosine phosphatase SHP2 (CHO-SHP2-C/S cells) (Noguchi *et al*., 1994). In contrast, a high concentration of LPA induced activation of MAP kinase in CHO-SHP2-C/S cells to an extent similar to that observed in parental CHO-IR cells (Takeda *et al*., 1998), suggesting that LPA and insulin may activate MAP kinase through different mechanisms. LPA could stimulate MAP kinase activation through a RAS-independent pathway in CHO-SHP2-C/S cells. To investigate this possibility further, we exposed CHO-IR cells or CHO-ΔSOS cells, which overexpress a mutant SOS protein that lacks a domain required for guanine nucleotide exchange and appears to act in a dominant-negative manner (Sakaue *et al*., 1995) to various concentrations of LPA or insulin for 5 min. Cell lysates were then subjected to immunoblot analysis with antibodies specific for tyrosine-phosphorylated (activated) MAP kinase. As previously described (Sakaue *et al*., 1995), unlike its marked effect in CHO-IR cells, insulin, even at concentrations as high as 100 nM, had little or no effect on MAP kinase activation in CHO-ΔSOS cells (Figure 1). The extent of MAP kinase activation in response to low concentrations (10–100 nM) of LPA was markedly reduced in CHO-ΔSOS cells relative to that in control CHO-IR cells, indicating that the effect of LPA at these low concentrations on MAP kinase activation is dependent on RAS. In contrast, the maximal effect of LPA on MAP kinase activation, observed at 1 μM LPA, was similar in both cell types (Figure 1). Similar results were obtained when MAP kinase activation was monitored with an *in vitro* kinase assay (data not shown).

Pre-treatment of CHO-ΔSOS cells with PTX inhibited MAP kinase activation in response to 1 μM LPA (Figure 2A), suggesting that a G_i protein (Moolenaar, 1995) may mediate this effect of LPA. We next investigated the effects of LPA on RAS activation in CHO-IR and CHO-ΔSOS cells by measuring the increase in the ratio of GTP-bound RAS to GDP-bound RAS. Both 100 nM insulin and 5 μM LPA induced RAS activation in CHO-IR cells, whereas neither agent exerted this effect in CHO-ΔSOS cells (Figure 2B). In addition, to exclude the possibility that LPA activates MAP kinase only in stable cell lines such as CHO-ΔSOS cells or CHO-SHP2-C/S cells, we examined the effect of LPA on MAP kinase activation in CHO-IR cells that were transiently transfected with a vector encoding a dominant-negative mutant of RAS (H-RAS- N17). Transient expression of H-RAS-N17 inhibited the insulin-induced activation of co-expressed hemagglutinin (HA) epitope-tagged MAP kinase, but it had virtually no effect on the response to LPA (Figure 2C). In addition, transient expression of a dominant-negative RAF (RAF-K375W) (Lopez-Ilasaca *et al*., 1997) also inhibited the insulin-induced activation of co-expressed HA epitope-tagged MAP kinase, but it caused only a small decrease of the response to LPA as compared with the control (Figure 2D). These results suggested that, in CHO cells, LPA acts through a PTX-sensitive G_i protein to activate MAP kinase in a manner independent of RAS or RAF.



Activation of MEK, but not of RAF1, by LPA in CHO-ΔSOS cells

We next determined whether LPA induces RAF1 or MEK activation in CHO-ΔSOS cells; RAF-1 is an upstream activator of MEK, and MEK activates MAP kinase. Whereas both LPA and insulin induced RAF1 activation in CHO-IR cells, neither exerted a substantial effect on RAF1 activity in CHO-ΔSOS cells (Figure 3A). In contrast, whereas both LPA and insulin induced MEK activation in CHO-IR cells, only LPA exhibited this effect in CHO-ΔSOS cells (Figure 3B). Pre-treatment of CHO-ΔSOS cells with PD98059, a specific inhibitor of MEK (Dudley *et al*., 1995), prevented the activation of both MEK and MAP kinase by LPA (Figure 3C), suggesting that LPA-induced activation of MAP kinase may be mediated by MEK, but not by RAF1, in CHO-ΔSOS cells.

Role of PI3Kγ in LPA-induced activation of MEK and MAP kinase in CHO-ΔSOS cells

Because PI3K has been implicated in G_i-mediated activation of MAP kinase (Hawes *et al*., 1996; Kranenburg *et al*., 1997), we next examined whether PI3K also contributes to LPA-induced MAP kinase activation in CHO-ΔSOS cells. Pre-treatment of CHO-ΔSOS cells with wortmannin, a PI3K inhibitor (Yano *et al*., 1993), inhibited LPA-induced activation of MAP kinase in a concentration-dependent manner (Figure 4A). Pre-treatment of parental CHO-IR cells with wortmannin also inhibited LPA-induced activation of MAP kinase (Figure 4A). However, the extent of inhibition of LPA-induced MAP kinase activity by wortmannin was less in CHO-IR cells than that observed in CHO-ΔSOS cells (Figure 4A). Pre-treatment of CHO-ΔSOS cells with wortmannin inhibited

Fig. 2. PTX-sensitive, RAS-independent activation of MAP kinase by LPA. (**A**) Effect of PTX on LPA-induced activation of MAP kinase in CHO-ΔSOS cells. Cells were pre-treated at 37°C for 24 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of PTX (100 ng/ml), and were then incubated with (lanes 2 and 4) or without (lanes 1 and 3) 1 μM LPA for an additional 5 min. Cell lysates were subjected to immunoblot analysis with α-P-MAPK or α-91. (**B**) Effects of LPA and insulin on RAS activation in CHO-IR (CHO) and CHO-ΔSOS (ΔSOS) cells. Cells were labeled with [^{32}P]orthophosphate, incubated for 5 min in the absence or presence of 100 nM insulin or 5 μM LPA, and then subjected to immunoprecipitation with the Y13-259 mAb to RAS. Bound nucleotides were eluted from the immunoprecipitates, separated by thin-layer chromatography, and detected by autoradiography (upper panel). The radioactivity incorporated into GTP and GDP was measured by image analysis, and the amount of RAS-GTP was expressed as a percentage of RAS-GTP plus RAS-GDP; data are means ± SE from three independent experiments (lower panel). (C and D) Effects of H-RAS-N17 (**C**) or a dominant-negative RAF (RAF-K375W) (**D**) on LPA- and insulin-induced activation of MAP kinase in CHO-IR cells. Cells were transiently transfected with 1 μg of pcDNA3 vector encoding HA epitope-tagged MAP kinase in the absence or presence of 4 μg of pSRα vector encoding H-RAS-N17 (Ras-DN) (C) or 4 μg of pSRα vector encoding a dominant-negative RAF (Raf-DN) (D). At 48 h after transfection, the cells were incubated for 5 min in the absence (–) or presence (+) of 100 nM insulin (Ins.) or 1 μM LPA, and cell lysates were then subjected to immunoprecipitation (IP) with mAb 12CA5 to HA (α-HA). The resulting immunoprecipitates were subjected to immunoblot analysis with α-P-MAPK (upper panel), and the same blot was also probed with α-HA (middle panel) to confirm that equal amounts of HA-MAP kinase (HA-p42) were present in each lane. Cell lysates were also subjected to immunoblot analysis with mAb to H-RAS (α-H-RAS) (C) or a polyclonal antibodies to RAF (D).

LPA-induced activation of MEK in a concentration-dependent manner (Figure 4B). Similarly, pre-treatment of CHO-ΔSOS cells with LY294002, another PI3K inhibitor (Vlahos *et al*., 1994), prevented LPA-induced activation of MAP kinase (Figure 4C). We have shown previously that overexpression of Δp85 (a dominant-negative mutant of the PI3K p85 adaptor subunit that lacks the binding site for the p110α or p110β catalytic subunit) markedly inhibits both the insulin-induced association of PI3K activity with IRS1 and insulin-stimulated glucose transport in CHO-IR cells (Hara *et al*., 1994). However, overexpression of Δp85 did not affect LPA-induced activation of MAP kinase in CHO-ΔSOS cells (Figure 4D).

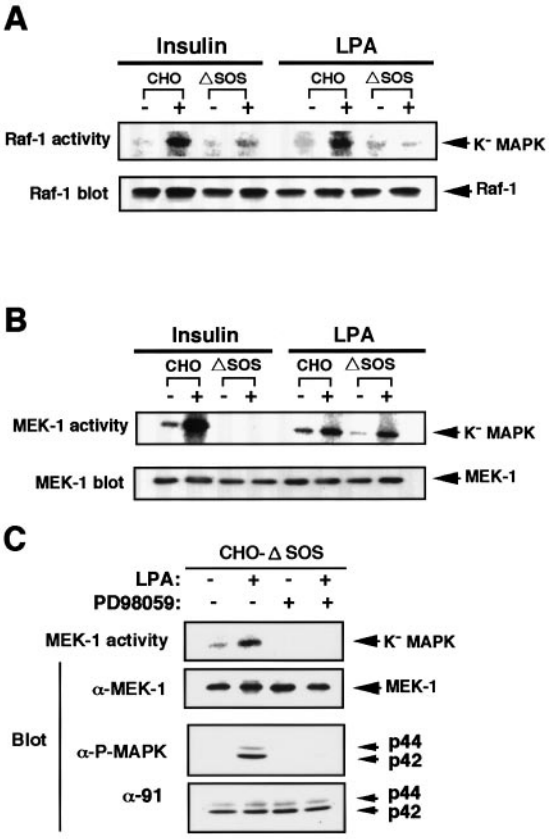


Fig. 3. Activation of MEK, but not of RAF1, by LPA in CHO-ΔSOS cells. (**A**) Effects of LPA and insulin on RAF1 activity in CHO-IR and CHO-ΔSOS cells. Cells were incubated for 5 min in the absence or presence of 100 nM insulin or 1 μM LPA. Cell lysates were then subjected to immunoprecipitation with polyclonal antibodies to RAF1, and kinase activity in the resulting precipitates was assayed, in the presence of a MEK fusion protein, with a kinase-defective MAP kinase (K^- MAPK) as ultimate substrate (upper panel). Duplicate immunoprecipitates were subjected to immunoblot analysis with polyclonal antibodies to RAF1 (lower panel). (**B**) Effects of LPA and insulin on MEK activity in CHO-IR and CHO-ΔSOS cells. Lysates of LPA- or insulin-stimulated cells were subjected to immunoprecipitation with polyclonal antibodies to MEK1, and kinase activity in the resulting precipitates was assayed with K^- MAPK as substrate (upper panel). Duplicate immunoprecipitates were also subjected to immunoblot analysis with polyclonal antibodies to MEK1 (lower panel). (**C**) Effect of PD98059 on LPA-induced activation of MEK and MAP kinase in CHO-ΔSOS cells. Cells were pre-treated with or without 50 μM PD98059 for 30 min, and then incubated for 5 min with or without 1 μM LPA. Cell lysates were assayed for MEK activity as in (B) (top panel), with duplicate immunoprecipitates subjected to immunoblot analysis with polyclonal antibodies to MEK1 (second panel). The cell lysates were also subjected to immunoblot analysis with antibodies specific for tyrosine-phosphorylated MAP kinase (α-P-MAPK) (third panel) and with α-91 polyclonal antibodies to p44 and p42 (bottom panel).

PI3Kγ (Stephens *et al*., 1994, 1997; Stoyanov *et al*., 1995), which is also sensitive to wortmannin, mediates G protein- and RAS-dependent activation of MAP kinase in COS-7 cells (Lopez-Ilasaca *et al*., 1997). Overexpression of wild-type PI3Kγ induced MAP kinase activation in CHO-ΔSOS cells in a manner dependent on the extent of overexpression (Figure 5A). In contrast, overexpression of wild-type PI3Kp110α failed to induce MAP kinase activation in CHO-ΔSOS cells (Figure 5A). Overexpression of the wild-type enzyme did not enhance further the LPA-induced activation of MAP kinase in CHO-ΔSOS cells, whereas that of a mutant PI3Kγ (K799R) that lacks lipid kinase activity and acts in a dominant-negative manner (Lopez-Ilasaca *et al*., 1997) markedly inhibited this action of LPA (Figure 5B). In parental CHO-IR cells, overexpression of a mutant PI3Kγ-K799R also markedly inhibited the LPA-induced activation of MAP kinase (Figure 5B). These data suggest that LPA-induced MAP kinase activation may be mediated by PI3Kγ in CHO-ΔSOS cells.

Role of atypical PKC in LPA-induced activation of MAP kinase in CHO-ΔSOS cells

We next examined whether PKC might contribute to LPA-induced MAP kinase activation in CHO-ΔSOS cells. Down-regulation of PKC induced by prolonged exposure of cells to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) inhibited MAP kinase activation in response to TPA but not that in response to LPA (Figure 6A). In addition, pre-treatment of CHO-ΔSOS cells with GO 6976, which inhibits conventional PKC isozymes but not other Ca^{2+}-independent isozymes (Martiny-Baron *et al*., 1993), did not affect LPA-induced MAP kinase activation (data not shown). In contrast, RO 31-8220, which at high concentrations (>1 μM) blocks the activation of atypical PKC isozymes (Ono *et al*., 1989; Nakanishi *et al*., 1993; Standaert *et al*., 1997) such as PKC-ζ and PKC-λ, inhibited activation of MEK and MAP kinase by LPA (Figure 6B), suggesting that atypical, but not conventional or novel, isoforms of PKC may play a role in this action of LPA in CHO-ΔSOS cells.

Thus, we next examined whether LPA stimulates the activity of PKC-ζ in CHO-ΔSOS cells. LPA increased PKC-ζ activity ~2-fold in both CHO-ΔSOS cells and parental CHO-IR cells (Figure 7A), and this effect was markedly inhibited by pre-treatment of CHO-ΔSOS cells with PTX (Figure 7A). Pre-treatment of CHO-ΔSOS cells with wortmannin also inhibited LPA-induced activation of PKC-ζ (Figure 7A), consistent with the notion that atypical PKC acts downstream of PI3K (Akimoto *et al*., 1996; Standaert *et al*., 1997). Overexpression of a kinase-negative mutant of PKC-ζ that previously has been shown to act in a dominant-negative manner (Díaz-Meco *et al*., 1994), but not that of the wild-type enzyme, substantially inhibited LPA-induced MAP kinase activation in CHO-ΔSOS cells (Figure 7B). Overexpression of the kinase-negative PKC-ζ did not affect MAP kinase activation in response to TPA (Figure 7B). Overexpression of a kinase-negative mutant of PKC-ζ also substantially inhibited LPA-induced MAP kinase activation in parental CHO-IR cells (Figure 7C). In addition, overexpression of wild-type

PI3Kγ also increased PKC-ζ activity ~2-fold, similar to the effect of LPA, in CHO-ΔSOS cells (Figure 7D). Moreover, the LPA-induced activation of PKC-ζ was markedly inhibited by overexpression of the dominant-negative PI3Kγ-K799R (Figure 7D). Together, these data suggest that a PI3Kγ-mediated increase in the activity of atypical PKC-ζ contributes to the activation of MAP kinase by LPA in CHO-ΔSOS cells.

Discussion

We have shown that LPA activates MAP kinase in a G_i-mediated, but RAS-independent, manner. This G_i-mediated effect of LPA appears to bypass RAF but still requires activation of MEK. Our observation that MAP kinase activation induced by low concentrations of LPA was markedly attenuated in CHO-ΔSOS cells suggests that RAS also contributes to this effect of LPA in CHO cells. However, RAS appears to be dispensable for activation of MEK and MAP kinase induced by high concentrations of LPA in these cells.

In contrast to our present observations, overexpression of either a dominant-negative murine SOS or RAS-N17 blocked the activation of MAP kinase in response to a high concentration of LPA (10 μM) in COS-7 cells (van Biesen *et al*., 1995; Luttrell *et al*., 1996), suggesting that heterogeneity in LPA signaling pathways may exist among cell types. The PTX-sensitive G_o protein α subunit activates MAP kinase in a RAS-independent manner in CHO cells, although this pathway is thought to involve a phorbol ester-sensitive PKC (van Biesen *et al*., 1996). In vascular smooth muscle cells, angiotensin II, which acts at G protein-coupled receptors, induces MAP kinase activation in a RAS-independent and PTX-insensitive manner, although this pathway is largely uncharacterized (Takahashi *et al*., 1997). In contrast to these pathways for MAP kinase activation, our present data indicate that the G_i protein-mediated, RAS-independent activation of MAP kinase by LPA involves PI3Kγ or a related enzyme.

As shown in the present study, LPA-induced MAP kinase activation is sensitive to PI3K inhibitors such as wortmannin or LY294002 (Hawes *et al*., 1996; Kranenburg *et al*., 1997). Whereas a dominant-negative mutant of the p85 subunit of PI3K was shown previously to inhibit activation of MAP kinase by LPA in COS-7 cells (Hawes *et al*., 1996), such an inhibitory effect was not apparent in CHO-ΔSOS cells. The reason for this discrepancy remains unclear; however, antibody microinjection experiments have indicated that the PI3Kp110α isozyme does not contribute to the mitogenic effect of LPA (Roche *et al*.,

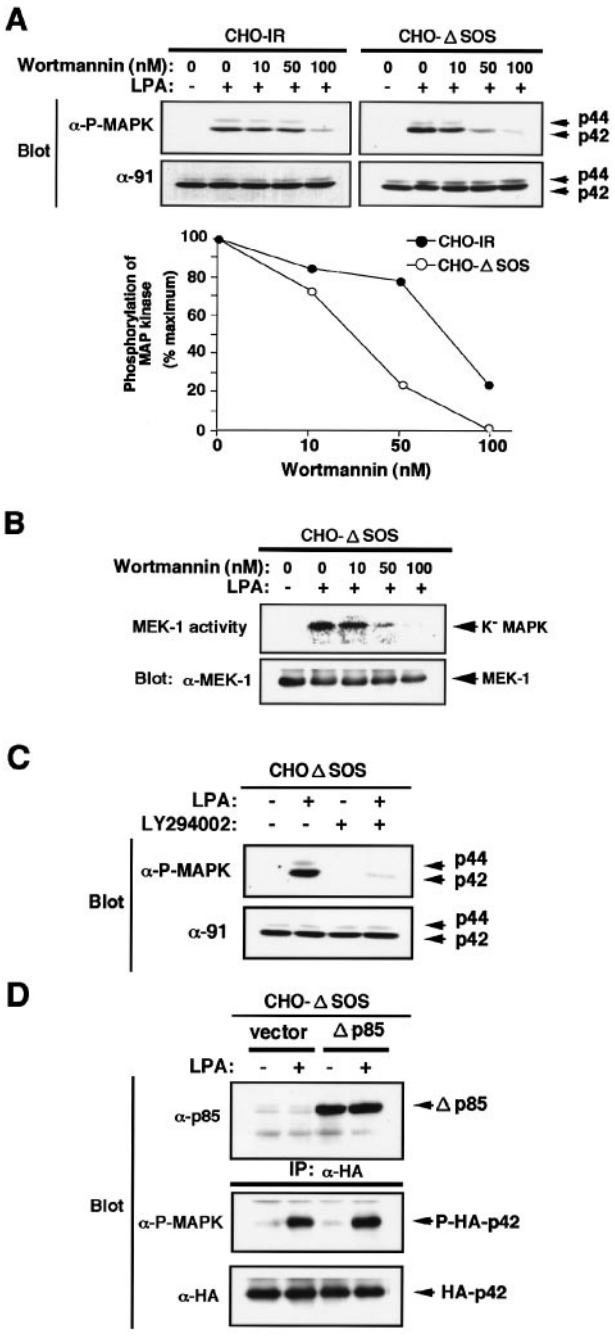


Fig. 4. Inhibition of LPA-induced activation of MEK and MAP kinase by PI3K inhibitors, but not by overexpression of a dominant-negative mutant of the p85 subunit of PI3K, in CHO-ΔSOS cells. (**A**) Effect of wortmannin on LPA-induced activation of MAP kinase. CHO-ΔSOS cells or CHO-IR cells were pre-treated with the indicated concentrations of wortmannin for 30 min, and were then incubated in the absence or presence of 1 μM LPA for 5 min. The activities of MAP kinase in cell lysates were then assayed as described in Figure 3C (upper panels). The extent of tyrosine phosphorylation of p42 MAP kinase was quantified by scanning densitometry with the NIH image program (lower panels). Data are expressed as a percentage of the maximal value, apparent with 1 μM LPA in parental CHO-IR cells, and are means of three independent experiments. (**B**) Effect of wortmannin on LPA-induced activation of MEK. CHO-ΔSOS cells were pre-treated with the indicated concentrations of wortmannin for 30 min, and were then incubated in the absence or presence of 1 μM LPA for 5 min. The activities of MEK in cell lysates were then assayed as described in Figure 3C. (**C**) Effect of LY294002 on LPA-induced MAP kinase activation. CHO-ΔSOS cells were pre-treated with or without 200 μM LY294002 for 30 min, and were then incubated in the absence or presence of 1 μM LPA for 5 min. The activity of MAP kinase in cell lysates was assayed as in (A). (**D**) Effect of overexpression of Δp85 on LPA-induced activation of MAP kinase. CHO-ΔSOS cells were transiently co-transfected with 1 μg of pcDNA3 vector containing HA-tagged MAP kinase cDNA and with either 4 μg of pSRα encoding Δp85 or with pSRα (vector) alone. The transfected cells were incubated with or without 1 μM LPA for 5 min, after which cell lysates were subjected to immunoprecipitation (IP) with mAb 12CA5 to HA. The resulting precipitates were subjected to immunoblot analysis with α-P-MAPK (middle panel) or, in order to determine the amount of HA-MAP kinase present, with α-HA (lower panel). Cell lysates were also subjected to immunoblot analysis with a mAb to PI3K p85 subunit (α-p85) in order to confirm equal expression of Δp85 (upper panel).

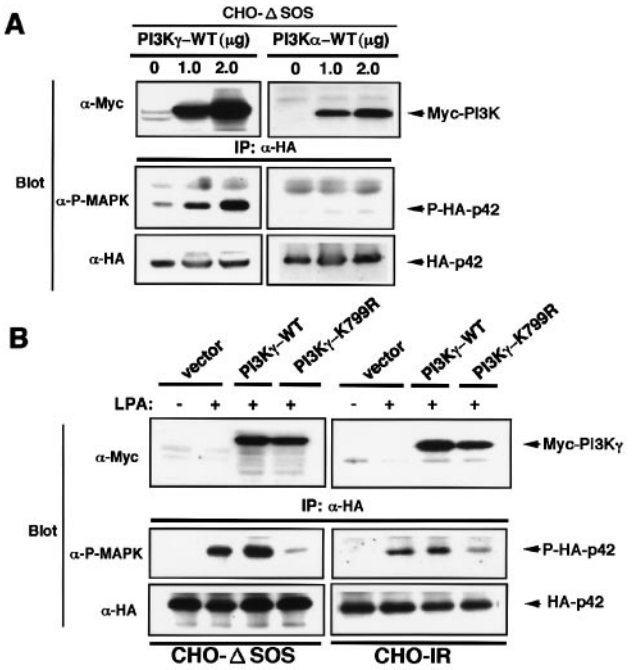


Fig. 5. Effects of overexpression of wild-type or dominant-negative PI3Kγ on LPA-induced activation of MAP kinase in CHO-ΔSOS cells. (**A**) Cells were transiently co-transfected with the indicated amounts of pcDNA3 vector encoding Myc epitope-tagged wild-type PI3Kγ (PI3Kγ-WT) (left panels) or SRα vector encoding Myc epitope-tagged wild-type PI3Kp110α (PI3Kα-WT) (right panels) and with 1 μg of pcDNA3 encoding HA-tagged MAP kinase. Lysates of transfected cells were subjected to immunoprecipitation with mAb 12CA5, and the resulting precipitates were subjected to immunoblot analysis with α-P-MAPK (middle panel) or with α-HA (lower panel). Cell lysates were also subjected to immunoblot analysis with the 9E10 mAb to Myc (α-MYC) in order to determine the amount of Myc-tagged PI3Kγ (upper left panel) or the amount of Myc-tagged PI3Kp110α (upper right panel). (**B**) CHO-ΔSOS cells (left panels) or parental CHO-IR cells (right panels) were transiently co-transfected with 1 μg of pcDNA3 encoding HA-tagged MAP kinase and 4 μg of pcDNA3 encoding either Myc-tagged PI3Kγ-WT or Myc-tagged PI3Kγ-K799R [or pcDNA3 (vector) alone]. The transfected cells were incubated with or without 1 μM LPA for 5 min, after which cell lysates were subjected to immunoprecipitation with mAb 12CA5. The resulting precipitates were subjected to immunoblot analysis with α-P-MAPK (middle panel) or with α-HA (lower panel). The cell lysates were also subjected to immunoblot analysis with mAb 9E10 to Myc (upper panel).

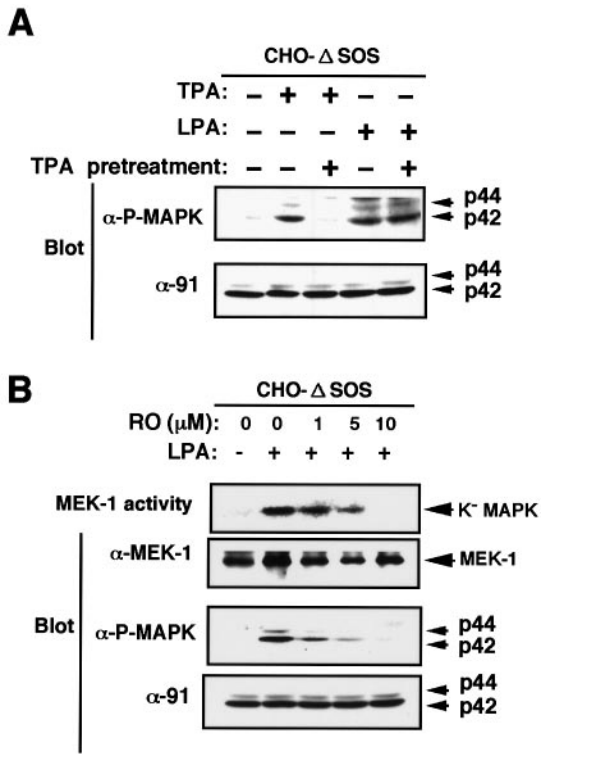


Fig. 6. Effect of TPA pre-treatment (**A**) or RO 31-8220 (**B**) on LPA-induced activation of MEK or MAP kinase in CHO-ΔSOS cells. (A) Cells were pre-treated for 24 h in serum-free medium in the absence or presence of 800 nM TPA, and were then incubated for 5 min with or without 200 nM TPA or 1 μM LPA, as indicated. Cell lysates were subjected to immunoblot analysis with α-P-MAPK (upper panel) or with α-91 polyclonal antibodies to p44 and p42 MAP kinase (lower panel). (B) Cells were pre-treated for 30 min with the indicated concentrations of RO 31-8220 (RO), and then incubated for 5 min with or without 1 μM LPA. The activities of MEK and MAP kinase in cell lysates were then assayed as described in Figure 3C.

1994). In addition, we demonstrated that overexpression of PI3Kp110α did not stimulate MAP kinase activation in CHO-ΔSOS cells. Furthermore, constitutively active PI3Kα did not activate MAP kinase in COS cells (Klippel *et al*., 1996) or CHO-IR cells (W.Ogawa and M.Kasuga, unpublished data), suggesting that PI3Kp110α does not mediate LPA-induced MAP kinase activation, at least in CHO cells. The PI3K p110γ isozyme was first isolated on the basis of the observation that its enzymatic activity is greatly increased by Gβγ subunits (Stoyanov *et al*., 1995; Stephens *et al*., 1997). It subsequently was implicated in the Gβγ-induced tyrosine phosphorylation of SHC, which results in the sequential activation of RAS, RAF and MAP kinase, in COS-7 cells (Lopez-Ilasaca *et al*., 1997). In the present study, overexpression of wild-type PI3Kγ alone induced MAP kinase activation in CHO-ΔSOS cells, and that of a dominant-negative mutant of PI3Kγ markedly inhibited LPA-induced MAP kinase activation. Thus, our data suggest that PI3Kγ mediates RAS-independent activation of MEK and MAP kinase in CHO cells, as well as RAS-dependent activation of these kinases in COS cells. Given that PI3Kγ is abundant in hematopoietic cells but not in other cell types, including CHO cells (H.Takeda, T.Matozaki and M.Kasuga, unpublished data), either the small amount of PI3Kγ in CHO cells is sufficient, or an unidentified isozyme related to PI3Kγ is responsible for the RAS-independent activation of MEK and MAP kinase.

We have also shown that LPA increases the activity of atypical PKC-ζ in CHO-ΔSOS cells, again in a PTX-sensitive manner. In addition, a dominant-negative mutant of PKC-ζ markedly inhibited LPA-induced MAP kinase activation, suggesting that PKC-ζ mediates, at least in part, RAS-independent activation of MAP kinase by LPA. In contrast, either down-regulation of PKC by prolonged exposure to TPA or inhibition of PKC by GO 6976 had no effect on LPA-induced MAP kinase activation in CHO-ΔSOS cells. Thus, conventional or novel PKC isoforms do not appear to be responsible for MAP kinase activation in response to LPA in these cells. Inhibition of PKC-ζ has been shown to attenuate mitogenesis induced by fetal bovine serum (FBS, the principle active component of which appears to be LPA) (Berra *et al*., 1993). In addition, PKC-ζ mediates MAP kinase activation in response to serum, tumor necrosis factor-α or angiotensin II (Berra *et al*., 1995; Liao *et al*., 1997). Consistent with the

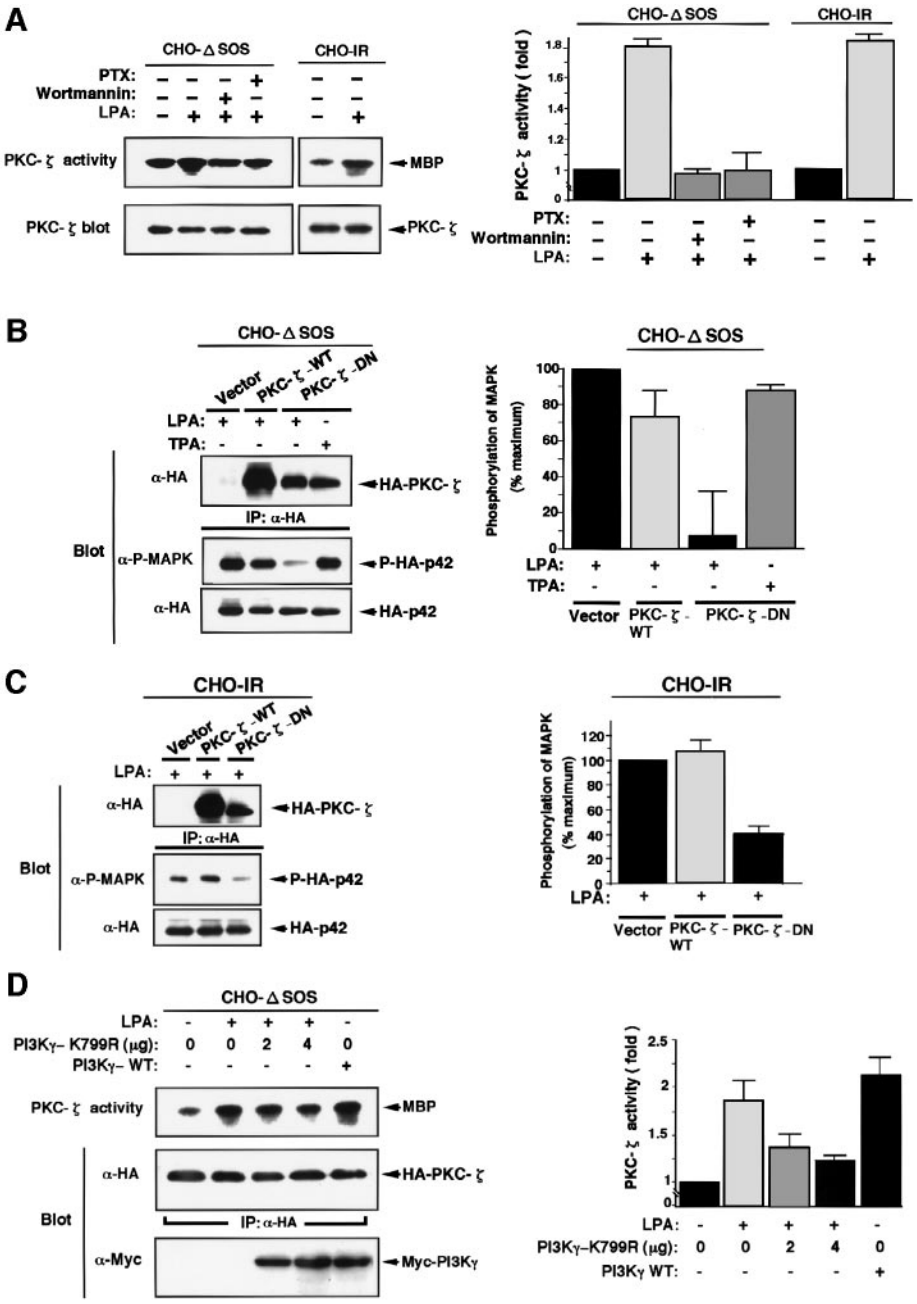


Fig. 7. Role of atypical PKC in LPA-induced activation of MAP kinase in CHO-ΔSOS cells. (**A**) Effects of PTX and wortmannin on LPA-induced activation of PKC-ζ. CHO-ΔSOS cells (left panel) or CHO-IR cells (right panel) were pre-treated in the absence or presence of PTX (100 ng/ml) for 24 h or of 100 nM wortmannin for 30 min, and were then incubated with or without 1 μM LPA for 5 min. Cell lysates were subjected to immunoprecipitation with polyclonal antibodies to PKC-ζ, and the resulting precipitates were assayed for kinase activity with MBP as substrate. Duplicate immunoprecipitates were also subjected to immunoblot analysis with polyclonal antibodies to PKC-ζ (left panels). The amount of radioactivity incorporated into MBP was measured by image analysis; PKC-ζ activity was expressed as fold increase (relative to that of cells not exposed to PTX, wortmannin or LPA), and data are means ± SE from three independent experiments (right panel). (B and C) Effects of overexpression of wild-type or dominant-negative PKC-ζ on LPA- or TPA-induced activation of MAP kinase. CHO-ΔSOS cells (**B**) or CHO-IR cells (**C**) were transiently co-transfected with 1 μg of pcDNA3 encoding HA-tagged MAP kinase and 4 μg of pRcCMV vector encoding either HA-tagged wild-type PKC-ζ (PKC-ζ-WT) or HA-tagged kinase-negative PKC-ζ (PKC-ζ-DN) [or pRcCMV alone (vector)]. The transfected cells were incubated with or without 1 μM LPA or 200 nM TPA for 5 min, after which cell lysates were subjected to immunoprecipitation with mAb 12CA5 to HA (α-HA). The resulting precipitates were then subjected to immunoblot analysis with α-P-MAPK (middle left panel) or with α-HA (lower left panel). The cell lysates were also subjected to immunoblot analysis with α-HA to determine the amount of HA-PKC-ζ (upper left panel). The phosphorylation of HA-tagged MAP kinase was also quantified by densitometry and expressed as a percentage of maximal value, apparent with 1 μM LPA in cells transfected with a vector alone (right panel); data are means ± SE from three independent experiments. (**D**) Effects of overexpression of wild-type or dominant-negative PI3Kγ on LPA-induced activation of PKC-ζ. Cells were transiently co-transfected with 1 μg of pRcCMV encoding HA-tagged wild-type PKC-ζ and pcDNA3 vector encoding either Myc-tagged wild-type PI3Kγ (PI3Kγ-WT) (4 μg) or Myc-tagged PI3Kγ-K799R (2 or 4 μg). The transfected cells were incubated with or without 1 μM LPA for 5 min, after which cell lysates were subjected to immunoprecipitation with α-HA. The resulting immunoprecipitates were assayed for the kinase activity of HA-PKC-ζ with MBP as substrate (upper left panel); duplicate immunoprecipitates were also subjected to immunoblot analysis with α-HA (middle left panel). Cell lysates were also subjected to immunoblot analysis with antibodies to Myc (lower left panel). The radioactivity incorporated into MBP was also measured by image analysis, and PKC-ζ activity was expressed as fold increase as indicated (right panel); data are means ± SE from three independent experiments.

recent observation that PKC-ζ directly phosphorylates and activates MEK, resulting in subsequent activation of MAP kinase (Díaz-Meco *et al*., 1994; Schönwasser *et al*., 1998), LPA induced activation of MEK, but not RAF1, in CHO-ΔSOS cells.

PKC-ζ has been suggested to function as a downstream effector of PI3K p110α or p110β isozymes in the activation of gene expression by growth factors (Akimoto *et al*., 1996) or in the stimulation of glucose transport by insulin (Standaert *et al*., 1997). LPA-induced PKC-ζ activation was sensitive to wortmannin in CHO-ΔSOS cells. Furthermore, overexpression of wild-type PI3Kγ increased PKC-ζ activity to a similar extent to LPA. In addition, a dominant-negative mutant of PI3Kγ abolished the increase in PKC-ζ activity induced by LPA in CHO-ΔSOS cells. Together, these data suggest that LPA induces PI3Kγ-dependent activation of atypical PKC-ζ, which then leads to activation of MEK and MAP kinase, in CHO-ΔSOS cells. To our knowledge, this is the first demonstration of activation of atypical PKC by PI3Kγ.

We have shown that wortmannin inhibits the LPA-stimulated MAP kinase activation in CHO-IR cells as well as in CHO-ΔSOS cells, although the extent of inhibition of LPA-induced MAP kinase activity by wortmannin in CHO-IR cells is less than that observed in CHO-ΔSOS cells. It is possible that the Ras-dependent MAP kinase activation induced by LPA may be less sensitive to wortmannin than the Ras-independent MAP kinase activation induced by LPA in CHO-IR cells. In addition, a dominant-negative mutant of PI3Kγ blocked LPA-induced MAP kinase activation in CHO-IR cells. PI3Kγ has been shown to mediate the G_i-induced MAP kinase activation which involves Ras (Lopez-Ilasaca *et al*., 1997). Thus, PI3Kγ may mediate both the Ras-dependent and -independent MAP kinase activation in CHO-IR cells. Furthermore, a dominant-negative PKC-ζ also inhibited LPA-induced MAP kinase activation in CHO-IR cells. Thus, the PI3Kγ- and atypical PKC-mediated pathway leading to MAP kinase activation is not peculiar to CHO-ΔSOS cell lines but does exist in parental CHO cells.

The mechanism by which PI3Kγ activates atypical PKC in response to LPA remains unclear. PI3Kγ may activate PKC-ζ by generating phosphatidylinositol 3,4,5-trisphosphate, which has been shown to be a potent and selective activator of PKC-ζ (Nakanishi *et al*., 1993). Protein kinase B (PKB), also known as AKT, is activated by a variety of extracellular stimuli and is another downstream effector of PI3K (Hemmings, 1997; Marte and Downward, 1997). Recently, a protein kinase, termed PKB kinase or PDK1, was identified that phosphorylates and activates PKB in the presence of lipid vesicles containing either phosphatidylinositol 3,4,5-trisphophate or phosphatidylinositol 3,4-bisphosphate (Alessi *et al*., 1997; Stephens *et al*., 1998). Thus, an intermediate kinase, such as PKB kinase, that is regulated by PI3Kγ might also be responsible for activation of PKC-ζ. It is also possible that the upstream region of the kinase domain of PI3Kγ could be actually interacting with PKC-ζ. Further studies will be required to characterize the entire pathway for G_i-mediated, RAS-independent activation of the MAP kinase cascade.

Materials and methods

Cells, vectors, antibodies and reagents

CHO-IR and CHO-ΔSOS cells were maintained in Ham's F-12 medium supplemented with 10% FBS as described previously (Noguchi *et al*., 1994; Sakaue *et al*., 1995). In several experiments, CHO-ΔSOS cells were incubated with PTX (100 ng/ml) (Sigma) or 800 nM TPA (Sigma) for 24 h as previously described (Hordijk *et al*., 1994; Takeda *et al*., 1998).

The cDNAs encoding H-RAS-N17, a dominant-negative mutant of RAF (RAF-K375W) (Lopez-Ilasaca *et al*., 1997), HA epitope-tagged wild-type rat PKC-ζ, HA-tagged kinase-defective PKC-ζ (Berra *et al*., 1995), a dominant-negative mutant (Δp85) of the p85 subunit of bovine PI3Kα (Hara *et al*., 1994), Myc epitope-tagged porcine PI3Kγ, Myc epitope-tagged bovine PI3Kp110α and HA-tagged p42 MAP kinase were incorporated into cytomegalovirus- or SV40-based expression plasmids. The mutant (K799R) PI3Kγ cDNA was generated by site-directed mutagenesis as described (Tsuda *et al*., 1998).

Monoclonal antibodies (mAbs) to the HA epitope or to the Myc epitope were used as culture supernatants from 12CA5 and 9E10 hybridomas (American Type Culture Collection), respectively. The F12 mAb to the p85 subunit of PI3Kα was prepared as described (Hara *et al*., 1994). Antibodies that react specifically with the tyrosine-phosphorylated form of MAP kinase were obtained from New England BioLabs. Rabbit polyclonal antibodies (α-91) to both p44 and p42 MAP kinase were prepared with a synthetic peptide corresponding to residues 307–327 of rat MAP kinase. Polyclonal antibodies to H-RAS or to RAF1 were obtained from Santa Cruz Biotechnology; polyclonal antibodies to PKC-ζ from Gibco-BRL; polyclonal antibodies to MEK-1 from UBI; and mAb to H-RAS from Transduction Laboratories. LPA, insulin, wortmannin and LY294002 were obtained from Sigma, and PD98059 was from New England BioLabs.

Transfection

Transient transfection of CHO-IR or CHO-ΔSOS cells was performed as described previously (Tsuda *et al*., 1998). Briefly, cells (~3×10^5 per 6 cm dish) were incubated for 5 h in 2 ml of serum-free medium containing 8 μl of Lipofectamine (Gibco-BRL) and the indicated amounts of various expression vectors. The transfection mixture was then supplemented with an equal volume of medium containing 20% FBS and incubated for an additional 19 h. The cells were washed and cultured in serum-free medium for 24 h before experiments. For transfection with the vector encoding the dominant-negative mutant of PKC-ζ, the transfected cells were cultured for 6 h after the addition of medium containing 20% FBS, and were then incubated for 12 h in serum-free medium before experiments.

Immunoprecipitation and immunoblot analysis

Subconfluent (~70%) cells (6 cm plates) were deprived of serum for 12 h, stimulated with LPA or insulin, washed with phosphate-buffered saline, and immediately frozen in liquid nitrogen. The cells were then lysed on ice in 0.5 ml of ice-cold lysis buffer [20 mM Tris–HCl (pH 7.6), 140 mM NaCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 1% (v/v) NP-40, 10% (v/v) glycerol] containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium vanadate, as previously described (Tsuda *et al*., 1998). The lysates were centrifuged at 10 000 *g* for 15 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation and immunoblot analysis. Briefly, supernatants were incubated for 4 h at 4°C with various antibodies bound to protein G–Sepharose beads (2 μg of antibody per 20 μl of beads) (Pharmacia), after which the beads were washed twice with 1 ml of WG buffer [50 mM HEPES–NaOH (pH 7.6), 150 mM NaCl, 0.1% (v/v) Triton X-100] and resuspended in SDS sample buffer. SDS–PAGE as well as immunoblot analysis with various antibodies and an ECL detection kit (Amersham) were performed as described previously (Takeda *et al*., 1998).

Immune complex kinase assays

The kinase activities of RAF1 and MEK were assayed as previously described (Chen *et al*., 1996). Briefly, immunoprecipitates prepared with polyclonal antibodies to RAF1 were incubated at 24°C for 15 min in 30 μl of kinase assay mixture containing 13.3 mM Tris–HCl (pH 7.5), 13.3 mM $MgCl_2$, 1.3 mM dithiothreitol (DTT), 32.5 μM ATP, 5 μCi of [γ-^{32}P]ATP (ICN) and 0.5 μg of a fusion protein of GST with wild-type MEK; after the addition of 2 μg of a GST fusion protein containing a kinase-defective MAP kinase (K^- MAPK), the mixture was incubated for an additional 15 min. Similarly, immunoprecipitates prepared with polyclonal antibodies to MEK1 were incubated at 24°C for 30 min in

40 µl of a kinase assay mixture containing 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 25 µM ATP, 5 µCi of [γ-^{32}P]ATP and 2 µg of the GST fusion protein containing the kinase-defective MAP kinase.

For assay of PKC-ζ activity, cells were lysed in a solution containing 50 mM Tris–HCl (pH 7.6), 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 0.5% Triton X-100, 10% glycerol, 2 mM DTT, 2 mM PMSF and 2 mM sodium vanadate. The lysates were then subjected to immunoprecipitation with polyclonal antibodies to PKC-ζ, or with the 12CA5 mAb in the case of CHO-ΔSOS cells transfected with a cDNA encoding HA-tagged wild-type PKC-ζ. The immune complexes were then incubated at 24°C for 15 min in 20 µl of kinase assay mixture containing 35 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM $CaCl_2$, 40 µM ATP, 0.5 µCi of [γ-^{32}P]ATP and 30 µM myelin basic protein (MBP).

All kinase reactions were terminated by addition of 20 µl of SDS sample buffer, and the amount of radioactivity incorporated into the phosphorylated substrate proteins was determined by electrophoresis and densitometry with a Fuji BAS 2000 image analyzer. MAP kinase activation was also monitored with a direct *in vitro* kinase assay as previously described (Noguchi *et al*., 1994).

Determination of RAS-GTP/Ras-GDP ratio

Measurement of the RAS-GTP/Ras-GDP ratio was determined as previously described (Noguchi *et al.*, 1994). Briefly, CHO-IR or CHO-ΔSOS cells were cultured in 6 cm dishes until confluence, and were then incubated in the absence of FBS for 20 h in Ham's F-12 medium containing 0.1% bovine serum albumin (BSA) and 10 mM HEPES–NaOH (pH 7.4). The cells subsequently were labeled for 3 h with 200 µCi of [^{32}P]orthophosphate (ICN) in 2 ml of phosphate-free RPMI 1640 (Gibco) supplemented with 0.1% BSA and 10 mM HEPES–NaOH (pH 7.4). After stimulation with insulin or LPA for 5 min, the cells were lysed in a solution containing 50 mM Tris–HCl (pH 8.0), 20 mM $MgCl_2$, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF and aprotinin (20 µg/ml). RAS was then immunoprecipitated with a specific mAb (Y13-259), and bound nucleotides were eluted in the presence of non-radioactive carrier. GDP and GTP were resolved by thin-layer chromatography on polyethyleneimine-cellulose plates with 1 M LiCl as solvent, and they were detected by autoradiography. The amount of radioactivity associated with GTP and GDP was determined by scanning the plate with a Fuji BAS 2000 image analyzer. The amount of GTP was expressed as a percentage of that of GDP plus GTP.

Expression and purification of recombinant MEK and kinase-negative MAP kinase

GST fusion proteins containing wild-type MEK or a kinase-defective MAP kinase (K^- MAPK) were generated as described previously (Noguchi *et al*., 1994). The GST fusion proteins were isolated from 0.5–2 l of bacterial culture by binding to glutathione–Sepharose beads (Pharmacia). Beads were washed twice with 10 ml of a solution containing 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA and 0.5% NP-40, and were then incubated for 30 min at 4°C with 7 ml of a solution containing 50 mM Tris–HCl (pH 9.6) and 10 mM glutathione (Sigma). Proteins eluted from the beads were dialyzed overnight against 1 l of a solution containing 25 mM Tris–HCl (pH 7.5), 1 mM EDTA and 1 mM DTT, and were then concentrated with a Centriprep-10 (Amicon) filtration device.

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