

# Involvement of phosphatidylinositol 3-kinase in the activation of extracellular signal-regulated kinase by PDGF in hepatic stellate cells

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**Abstract** Phosphatidylinositol 3-kinase (PI 3-K) is a lipid and protein kinase which associates with the activated platelet-derived growth factor (PDGF) receptor and other tyrosine kinases. We studied the effects of wortmannin, a selective inhibitor of PI 3-K, on the activation of extracellular-signal regulated kinase (ERK) by PDGF in cultured hepatic stellate cells, mesenchymal cells responsible for extracellular matrix synthesis within the liver. Incubation with 100 nM wortmannin, a dose which almost completely blocks PI 3-K, resulted in 50% reduction of ERK activity. Direct inhibition of ERK by wortmannin could not be considered responsible for this effect, since wortmannin did not inhibit ERK activity *in vitro*. Rather, inhibition of PI 3-K acts on the kinase cascade that leads to ERK activation, since PDGF-dependent phosphorylation of ERK was found to be reduced after incubation with wortmannin. Wortmannin also inhibited the increase in *c-fos* mRNA induced by PDGF, which is dependent on ERK activation. The results of this study show that in hepatic stellate cells PI 3-K is involved in ERK activation, although it is not necessary. These data indicate cross-talk between PI 3-K and the Ras/ERK pathway in PDGF-stimulated cells.

**Key words:** Extracellular signal-regulated kinase; Phosphatidylinositol 3-kinase; Wortmannin; Hepatic stellate cell; *c-fos*; Mitogen-activated protein kinase

## 1. Introduction

Platelet-derived growth factor (PDGF) regulates several cellular functions, such as mitogenesis, migration, and neoplastic transformation. PDGF signals through two tyrosine kinase receptors which dimerize and become phosphorylated on tyrosine residues upon binding to their ligand [1]. The phosphotyrosines on the activated receptor function as high-affinity binding sites for several molecules involved in downstream transmission of the signal, which bind through src-homology-2 (SH-2) domains or phosphotyrosine-binding domains [2,3]. Association of the PDGF receptor with the adapter protein Grb2 leads to recruitment of the exchange factor mSos and subsequent activation of Ras [4]. Activated Ras triggers a kinase cascade, with sequential activation of Raf-1, MEK and extracellular-signal regulated kinase (ERK) [5]. ERK belongs to the family of mitogen-activated protein kinases [5]. Two isoforms have been identified, and are referred to as ERK-1 (or p44<sup>MAPK</sup>) and ERK-2 (or p42<sup>MAPK</sup>) [6]. Upon translocation to the nucleus, ERK phosphorylates several transcription factors, including Elk-1 and SAP-1 [7–9]. Growth factor-mediated activation of

ERK has been shown to be an absolute requirement for triggering a proliferative response [10].

Another molecule which is recruited by the activated PDGF receptor is phosphatidylinositol 3-kinase (PI 3-K). This lipid and protein kinase is comprised of a 85-kDa regulatory subunit, equipped with two SH-2 domains, and a catalytic 110 kDa subunit [11]. PDGF stimulation leads to an association of PI 3-K with the activated receptor, and to tyrosine phosphorylation of p85 but not of p110. The signals generated by PI 3-K are only partially known, but this pathway has been shown to be sufficient to transduce a PDGF-dependent mitogenic signal [12]. Recent data indicate that the Ras/ERK and PI 3-K pathways may interact at different levels. Rodriguez-Viciana et al. [13] have reported that PI 3-K is a target of Ras. On the other hand, a mutant PDGF receptor that no longer associates with PI 3-K does not activate Ras [14], and transfection of a constitutively active form of PI 3-K results in stimulation of the Ras/ERK pathway [15]. These reports suggest that PI 3-K may act both downstream and upstream of Ras. However, no studies have yet elucidated the contribution of the PI 3-K pathway to the activation of ERK by PDGF in non-transformed, non-transfected cells. To address this issue, we used wortmannin, a selective PI 3-K inhibitor, which allows to study the effects of the interruption of the PI 3-K pathway in intact cells [16]. The effects of wortmannin on ERK activation in response to PDGF were investigated in hepatic stellate cells (HSC). HSC are liver-specific pericytes located in the hepatic sinusoid, which during liver injury undergo transition to a highly proliferative, matrix-producing phenotype and participate in the process of liver tissue healing and liver fibrosis [17]. PDGF is the most potent mitogen for human HSC in culture, and may be produced by HSC themselves, creating an autocrine loop [18]. Therefore, these cells represent an optimal system model to study the post-receptor effects of PDGF or other cytokines on primary cells, and the findings are potentially relevant for the pathophysiology of liver diseases. We report that PI 3-K is involved in PDGF-dependent activation of ERK, thus showing a cross-talk between PI 3-K and the Ras/ERK pathway in PDGF-stimulated cells.

## 2. Experimental

### 2.1. Reagents

Monoclonal, agarose-conjugated antiphosphotyrosine antibodies were purchased by Oncogene Science (Uniondale, NY, USA). Rabbit antisera against ERK were a kind gift of Dr. M.J. Dunn (Medical College of Wisconsin, USA). The antisera recognized both ERK-1 and ERK-2. Wortmannin, phosphatidylinositol, and myelin basic protein were from Sigma Chemical Co. (St. Louis, MO, USA). Protein A-Sepharose was purchased from Pharmacia (Uppsala, Sweden). [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) and [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) were from New England Nuclear (Milan, Italy). Human recombinant PDGF BB

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was from Boehringer Mannheim (Mannheim, Germany). All other reagents were of analytical grade.

## 2.2. Cell culture

Human liver HSC were isolated from liver tissue unsuitable for transplantation by collagenase/pronase digestion and centrifugation on stractan gradients, as previously described [19]. The cells were grown in Iscove's medium in the presence of 17% fetal calf serum.

## 2.3. Preparation of cell lysates

Confluent HSC were serum-starved for 48 h, and treated as indicated in section 3. They were then quickly placed on ice and washed with ice-cold PBS. The monolayer was lysed in RIPA buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% (w/v) aprotinin). Insoluble proteins were discarded by high-speed centrifugation at 4°C. Protein concentration in the supernatant was measured in triplicate using a commercially available assay (BioRad, Hercules, CA).

## 2.4. Phosphatidylinositol 3-kinase assay

PI 3-K assay was performed as described elsewhere [20]. Briefly, the cells were lysed in RIPA buffer and identical amounts of protein were immunoprecipitated using antiphosphotyrosine antibodies. After washing, the immunobeads were resuspended in 50  $\mu\text{l}$  of 20 mM Tris-HCl pH 7.5, 100 mM NaCl, and 0.5 mM EGTA. 0.5  $\mu\text{l}$  of 20 mg/ml phosphatidylinositol was added, and the samples were incubated at 25°C for 10 min. 1  $\mu\text{l}$  of 1 M  $\text{MgCl}_2$  and 10  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP were then added simultaneously, and the incubation was continued for 10 more minutes. The reaction was stopped by addition of 150  $\mu\text{l}$  of chloroform/methanol/37% HCl (10:20:0.2). Samples were extracted with chloroform and dried. Radioactive lipids were separated by thin-layer chromatography, developing with chloroform/methanol/30% ammonium hydroxide/water (46:41:5:8). After drying, the plates were autoradiographed. Identity of the 3-OH phosphorylated lipids after separation by thin-layer chromatography has been previously assessed by high-pressure liquid chromatography [20]. The radioactive spots were then scraped and counted.

## 2.5. Extracellular-signal regulated kinase assay

ERK was measured as the myelin basic protein kinase activity of ERK immunoprecipitates. RIPA lysates from HSC (60–100  $\mu\text{g}$  of protein) were immunoprecipitated with rabbit polyclonal anti-ERK antibodies and protein A-Sepharose. After washing, the immunobeads were incubated in a buffer containing 10 mM HEPES pH 7.4, 20 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 1 mM  $\text{Na}_3\text{VO}_4$ , 1  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP, and 0.5 mg/ml myelin basic protein for 30 min at 30°C. At the end of the incubation, 15  $\mu\text{l}$  of the reaction were spotted onto phosphocellulose disks, washed four times in 1% phosphoric acid, and the radioactivity counted in a beta-counter. Another aliquot (10  $\mu\text{l}$ ) of the reaction mixture was run on 15% SDS-PAGE [21]. After electrophoresis, the gel was dried and autoradiographed.

## 2.6. Western blotting

Identical amounts of protein were separated by SDS-PAGE according to Laemmli [21], and electroblotted on a polyvinylidene-difluoride membrane. The membranes were blocked overnight at 4°C with 2% bovine serum albumin in 0.1% PBS-Tween, and sequentially incubated at room temperature with rabbit anti-ERK antibodies and then horseradish peroxidase-conjugated anti-rabbit antibody (Gibco-BRL, Grand Island, NY, USA). Detection was carried out using chemiluminescence according to the manufacturer's protocol (Amersham, Arlington Heights, IL, USA).

## 2.7. Northern blot analysis

RNA was isolated according to Chomczynski and Sacchi [22]. Ten  $\mu\text{g}$  of total RNA were fractionated by 1% agarose-formaldehyde gel electrophoresis, and blotted on a nylon membrane. Procedures for DNA labeling and filter hybridization have been described in detail elsewhere [23]. Autoradiographic signals have been quantitated using a densitometer (BioRad, Hercules, CA, USA).

## 3. Results and discussion

We first tested the ability of wortmannin of blocking PDGF-

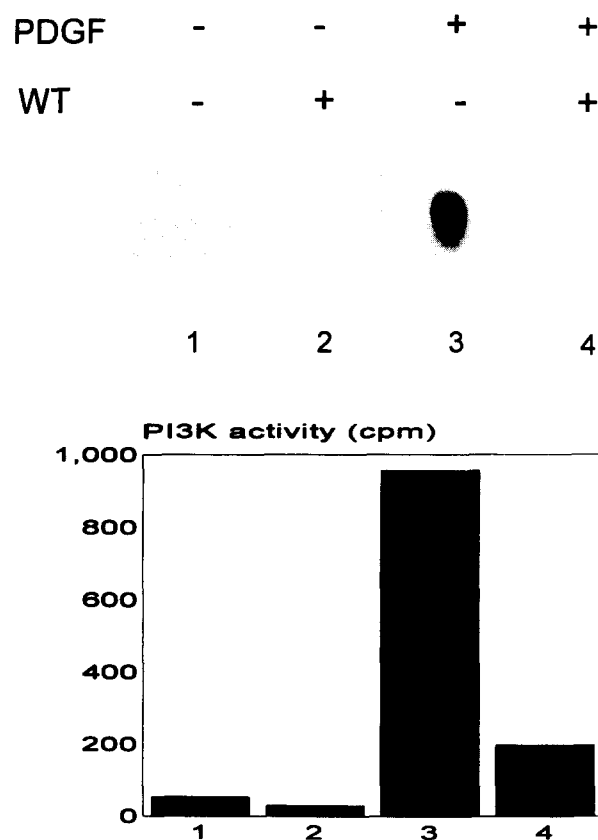


Fig. 1. Wortmannin blocks activation of PI 3-K by PDGF. Serum-starved HSC were incubated with 100 nM wortmannin (WT; lanes 2 and 4) or vehicle (dimethyl sulfoxide, lanes 1 and 3) for 30 min before addition of PDGF (10 ng/ml, lanes 3 and 4) for 10 min. Cell lysates were immunoprecipitated with antiphosphotyrosine antibodies and PI 3-K assay was carried out as described in section 2. The upper panel shows autoradiography of [ $^{32}\text{P}$ ]phosphatidylinositol-3-phosphate. The radioactive spots were scraped and counted in a beta-counter, as shown in the lower panel.

stimulated PI 3-K activity. Little PI 3-K activity was detectable in unstimulated, serum-starved HSC (Fig. 1). Incubation with PDGF (10 ng/ml) dramatically increased the PI 3-K activity associated with phosphotyrosine immunoprecipitates (Fig. 1, lane 3). Pretreatment of HSC with 100 nM wortmannin, resulted in nearly total inhibition of this activity (Fig. 1, lanes 3 and 4). Exposure of HSC to PDGF also stimulated ERK activity, which was increased 5 min following addition of the agonist, peaked at 10–15 min and persisted for at least 30 min (data not shown). Fig. 2A illustrates the effects of wortmannin on PDGF-stimulated ERK activity. Incubation with the PI 3-K inhibitor resulted in 50% inhibition of ERK activity (Fig. 2A, compare lanes 3 and 4). This incomplete inhibition was consistently observed in different experiments. However, a lower sensitivity of ERK to wortmannin cannot be considered responsible for this incomplete effect since doses as high as 0.5  $\mu\text{M}$  wortmannin resulted in an identical degree of inhibition (data not shown). Doses in the micromolar range were not used since they are no longer specific for PI 3-K [24]. Wortmannin did not affect ERK activity in unstimulated HSC (Fig. 2A, lane 2).

Although the effects of wortmannin at nanomolar concentrations are presumed to be specific for PI 3-K, a direct effect on ERK cannot be ruled out. Therefore, we studied whether wort-

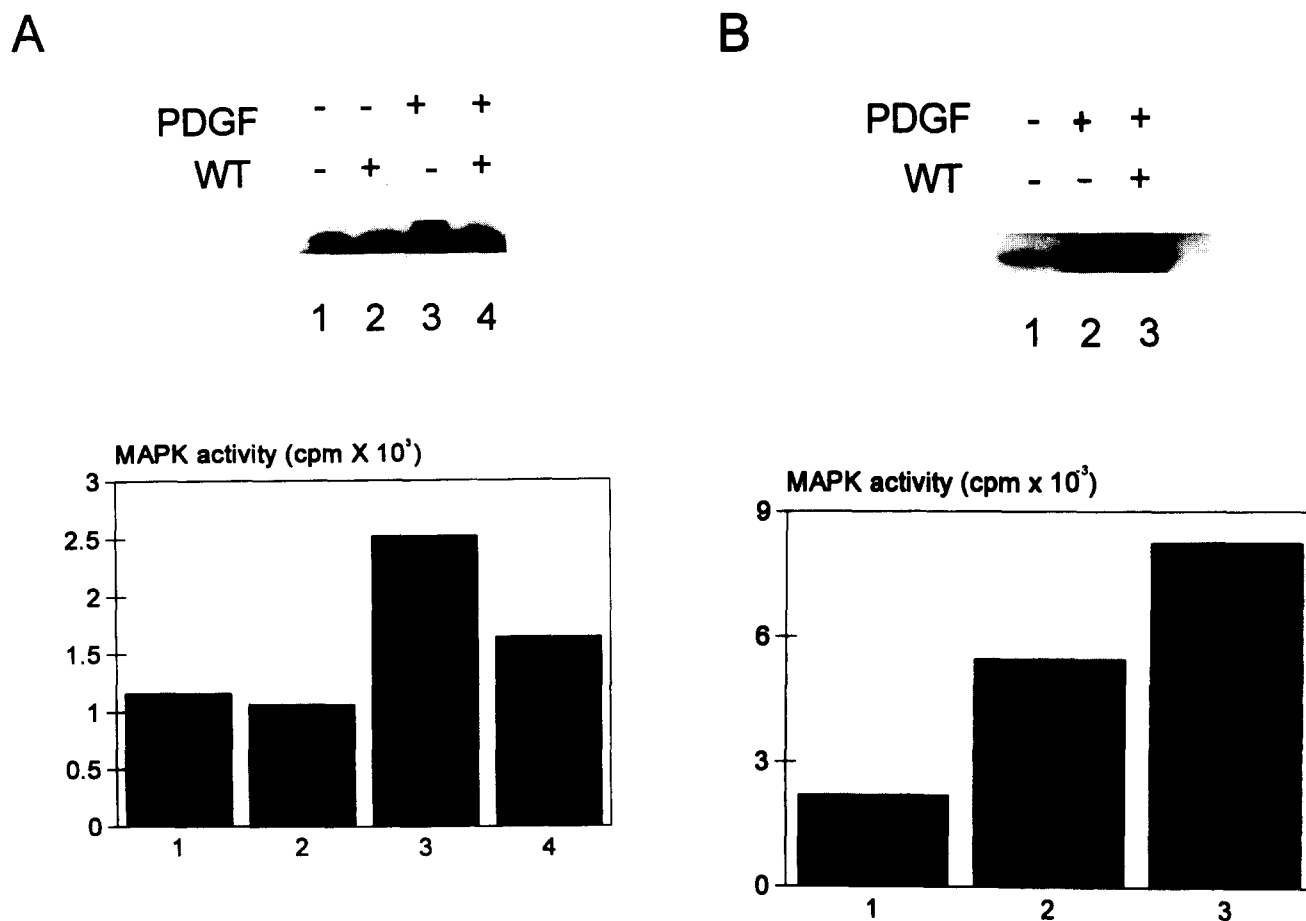


Fig. 2. Wortmannin inhibits ERK activation in vivo but not in vitro. (A) Serum-starved HSC were incubated exactly as described in Fig. 1. Cell lysates were immunoprecipitated with polyclonal anti-ERK antibodies, and the immunobeads were assayed for myelin basic protein kinase activity as described in section 2. The upper panel shows phosphorylated myelin basic protein after running on 15% SDS-PAGE. The lower panel shows counts after spotting the reaction mix on phosphocellulose discs and extensive washing. (B) Serum-starved HSC were incubated with 10 mg/ml PDGF (lanes 2 and 3). Cell lysates were immunoprecipitated with polyclonal anti-ERK antibodies. The immunobeads were assayed for ERK activity after addition of wortmannin (final concentration 100 nM, lane 3) or its vehicle (dimethyl sulfoxide, lanes 1 and 2). ERK assay was carried out as described in Fig. 2A. The small stimulatory effect in samples treated with wortmannin was not reproducible.

mannin is an inhibitor of ERK in vitro. HSC were incubated with PDGF and ERK assay was carried out after addition of 100 nM wortmannin or its vehicle (dimethyl sulfoxide) to the immunobeads (Fig. 2B). No differences in ERK activity were observed in the presence or absence of wortmannin (Fig. 2B). These data indicate that wortmannin does not exert a direct inhibitory effect on ERK, but that rather it blocks the pathways leading to its activation. Activation of ERK is regulated via phosphorylation through a cascade of different protein kinases, which constitute the so-called Ras/ERK pathway [5]. Stimulation with different growth factors, including PDGF, results in activation of Ras, which recruits the serine/threonine kinase Raf-1 to the plasma membrane, where it becomes active [25]. Raf-1 phosphorylates and activates a dual specificity kinase (ERK kinase, or MEK), which phosphorylates ERK on tyrosine and threonine residues [26,27]. Therefore, we investigated whether the observed inhibition of ERK by wortmannin was dependent on an effect on ERK phosphorylation. Exposure of HSC to PDGF resulted in a slower electrophoretic mobility of ERK-1 and ERK-2, indicating phosphorylation of tyrosine and/or threonine residues (Fig. 3, lanes 1 and 2). In the presence

of wortmannin, the amount of protein shifted upward was reduced, indicating reduced phosphorylation of both ERK isoforms (compare lane 2 and lane 3). As observed with the kinase assay (Fig. 2B), the inhibitory effect by wortmannin on ERK phosphorylation was incomplete, and this occurred even when higher doses (0.5  $\mu$ M) were employed (data not shown). Together, these data indicate that PI 3-K acts on the kinase cascade responsible for ERK activation.

In mammalian cells, the Ras/ERK pathway directs signals to the serum response element of the promoter of *c-fos* and other coregulated genes. Targets for ERK activity include the ternary complex factor (TCF) proteins Elk-1 and SAP-1 [8,28]. A TCF

Table 1  
Quantitative analysis of PDGF-induced *c-fos* expression in the presence or absence of wortmannin (data from Fig. 4)

	Control	PDGF 30 min	PDGF 60 min
Wortmannin	-	-	+
	0	4.25	2.96
		0.49	0.08

Data are the ratio of *c-fos*/36B4 mRNA abundance as measured by densitometry (arbitrary units).

protein binds to the serum response element along with the serum response factor and mediate increased gene expression [29]. We evaluated the effects of wortmannin on PDGF-induced increase in *c-fos* expression, as a target of ERK activation. The abundance of *c-fos* mRNA was increased 30 min after addition of PDGF and declined thereafter (Fig. 4). Pretreatment of HSC with wortmannin resulted in reduced *c-fos* expression at both time points (31% inhibition at 30 min, see Table 1), indicating inhibition of the nuclear phase of the Ras/ERK pathway. It is worth noticing that also in this case wortmannin reduced, but did not abolish, the increase in *c-fos* expression.

Growth factor-dependent signals are important for several disease states, such as hepatic or renal fibrosis, atherosclerosis or cancer. PDGF is a potent mitogen for HSC, and its expression is increased during liver injury [30]. PDGF activates PI 3-K, but the signal transduction pathways that originate from activation of this enzyme have only partially been clarified. PI 3-K regulates several cellular functions, including mitosis, cell migration, and programmed cell death [12,31,32]. Protein kinase C $\zeta$  [33], ribosomal S6 kinase [34], and the protooncogene c-Akt [35] have been indicated as downstream targets of the PI 3-K pathway. Data presented in this paper indicate that in non-transformed, non-transfected cells PI 3-K activation participates in PDGF-mediated activation of ERK. However, ERK activation appears to occur via both PI 3-K-dependent and independent pathways. In fact, a dose of wortmannin that virtually blocks PI 3-K, resulted in only 50% reduction of ERK activity in PDGF-stimulated cells. These observations are in keeping with data showing that activation of the Ras/ERK pathway occurs through Grb2/mSos [4]. According to the present study, concomitant activation of PI 3-K would lead to a greater activation of ERK, most likely acting at the level of Ras [15]. PI 3-K activation has been shown to be involved in PDGF's mitogenic signaling [12]. Preliminary data from our laboratory show that inhibition of PI 3-K using wortmannin

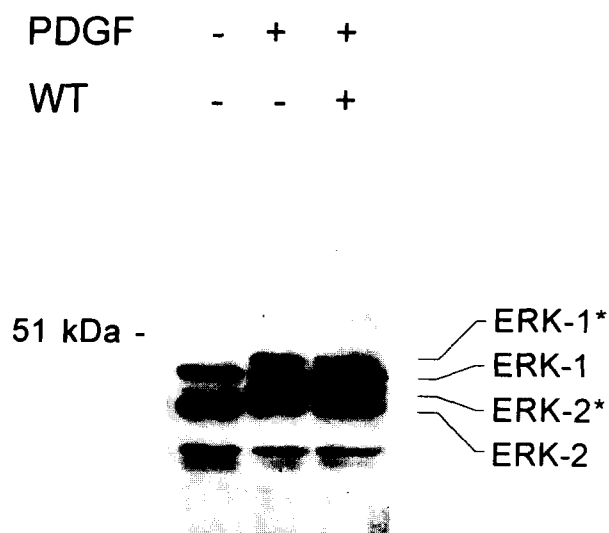


Fig. 3. Wortmannin inhibits ERK phosphorylation. Serum-starved HSC were incubated with 100 nM wortmannin (WT; lane 3) or vehicle (dimethyl sulfoxide, lanes 1 and 2) for 30 min before addition of PDGF (10 ng/ml, lanes 2 and 3) for 10 min. Twenty  $\mu$ g of cell lysate were separated by 12% SDS-PAGE and blotted with polyclonal anti-ERK antibodies. Bands corresponding to phosphorylated ERK are indicated by an asterisk (\*).

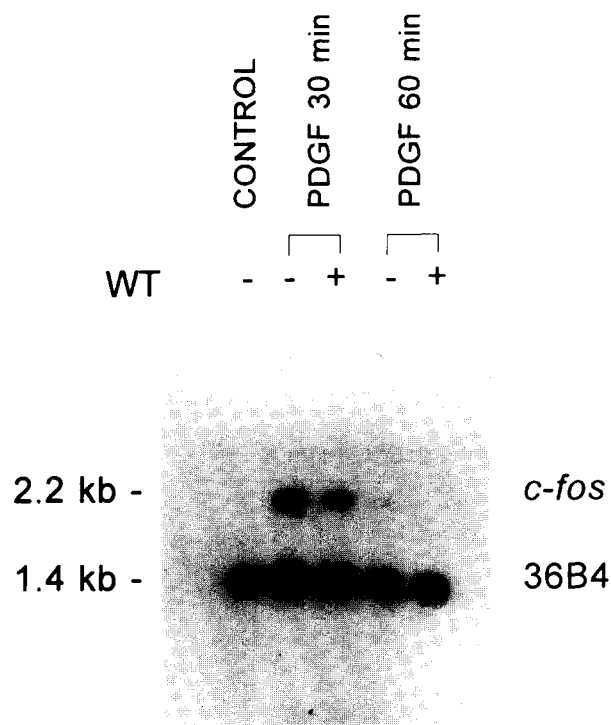


Fig. 4. Wortmannin inhibits PDGF-induced expression of *c-fos* mRNA. Serum-starved HSC were incubated with 100 nM wortmannin (WT) or vehicle (dimethyl sulfoxide) for 30 min before addition of PDGF (10 ng/ml) for the indicated time points. Ten  $\mu$ g of total RNA were separated on an agarose-formaldehyde gel, blotted on a membrane and hybridized with radiolabeled probes encoding for *c-fos* and for the ribosomal protein 36B4 (control gene).

completely blocks DNA synthesis in response to PDGF [36]. Since the inhibition of ERK activation by wortmannin is only partial, data from this study indicate that the mitogenic signal of PI 3-K follows ERK-dependent and independent pathways.

Tyrosine kinase receptors activate an array of different signaling pathways. Although they may be thought of as independent avenues leading to separate effects, accumulating evidence, including the data from this study, indicates cross-talk at different levels. Proliferation of HSC is a growth-factor regulated event, which is implicated in the deterioration of liver function during chronic disease [17]. A better understanding of the mechanisms that regulate the function of these effector cells will aid in the development of new strategies for the treatment of liver diseases.

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