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Differential involvement of ERK_{1-2} and $p38^{MAPK}$ activation on Swiss 3T3 cell proliferation induced by prostaglandin $F_{2\alpha}$

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Abstract **Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induces cyclin D_1 expression and DNA synthesis in Swiss 3T3 cells. In order to assess which signaling mechanisms are implicated in these processes, we have used both a pharmacological approach and interfering mutants. We demonstrate that $PGF_{2\alpha}$ induces extracellular-signal-regulated kinase (ERK_{1-2}) and $p38^{MAPK}$ activation, and inhibition of any of these signaling pathways completely blocks $PGF_{2\alpha}$-stimulated DNA synthesis. We also show that ERK_{1-2}, but not $p38^{MAPK}$ activation is required to induce cyclin D_1 expression, strongly suggesting that the concerted action of cyclin D_1 gene expression and other events are required to induce complete phosphorylation of retinoblastoma protein and S-phase entry in response to $PGF_{2\alpha}$.**
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Keywords: Prostaglandin $F_{2\alpha}$; MAPK; ERK_{1-2}; $p38^{MAPK}$; Cyclin D1; S-phase

1. Introduction

Mammalian cells proliferation is a highly coordinated phenomenon generally regulated by growth factors and extracellular matrix adhesion [1–4]. Most growth factors bind and activate receptors of tyrosine kinase, triggering the activation of specific signal transduction pathways. These mitogen-induced signals concertedly modulate the initiation of DNA replication and cell division, and this occurs by triggering a cascade of events that ultimately induce the expression of G_1 cyclins, key molecules that regulate crucial cell cycle transitions [5–7]. Several lines of evidence support the idea that cyclin Ds exert the main control on the transition of G_0 to S phase [6,8], and that most growth factors control G_1 phase progression by triggering the expression of cyclin Ds [9,10].

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulates DNA synthesis and proliferation of cultured Swiss mouse 3T3 cells [11], and is implicated in unrestricted multiplication of transformed cells [12]. Our previous work reveals that several $PGF_{2\alpha}$-triggered signaling events are required to induce cellular entry into S-phase, including increases in diacylglycerol, inositol 1,4,5-trisphosphate, intracellular Ca^{2+} ion mobilization, and protein kinase C (PKC) activation [11,13]. We have also shown that $PGF_{2\alpha}$ induction of cyclin D_1 expression plays a pivotal role in the control of DNA replication and the $PGF_{2\alpha}$-triggered cyclin D_1 expression involves a PKC-independent event, since $PGF_{2\alpha}$ is able to increase cyclin D_1 mRNA/protein levels in PKC-depleted cells [14]. Such a PKC-independent process may correspond to other early $PGF_{2\alpha}$-triggered events and both PKC-dependent and independent signals appear to be concertedly required for cells to initiate DNA synthesis. Furthermore, $PGF_{2\alpha}$ appears to induce DNA synthesis via the combined actions of the induction of cyclin D_1 gene expression and other signaling pathway-triggered events [14]. Thus, a basic question regarding $PGF_{2\alpha}$ signaling mechanisms is whether, and how, each personalized $PGF_{2\alpha}$ signal regulates cyclin D_1 expression, and how such events and others can ultimately control initiation of DNA synthesis.

Here we report that $PGF_{2\alpha}$ causes extracellular-signal-regulated kinase (ERK_{1-2}) and $p38^{MAPK}$ activation in Swiss 3T3 cells. Using a pharmacological approach as well as stable transfected cells with a dominant negative mutant of $p38^{MAPK}$ we determined that ERK_{1-2} and $p38^{MAPK}$ activation are essential for $PGF_{2\alpha}$-stimulated cellular entry into S-phase. We also show that ERK_{1-2} but not $p38^{MAPK}$ activation, is an essential event required to induce cyclin D_1 expression, suggesting that $p38^{MAPK}$ activation is involved in eliciting another process different from cyclin D_1 gene expression required to induce DNA synthesis in response to $PGF_{2\alpha}$.

2. Materials and methods

2.1. Cell culture

Swiss mouse 3T3 cells [15] were grown in DMEM containing 10% (v/v) fetal calf serum. Sub-confluent cultures were grown in 100-mm dishes at 37 °C equilibrated with 10% (v/v) CO_2.

2.2. Initiation of DNA synthesis assay

DNA synthesis analysis was performed as previously described [4]. Briefly, 1.5×10^5 cells were seeded in 35 mm dishes and growth until confluent and quiescent (6–8 days). Then cells were stimulated by addition of

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Abbreviations: $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$; ERK, extracellular-signal-regulated kinase; MAPK, mitogen activated protein kinase; pRb, retinoblastoma protein; FBS, fetal bovine serum; LIF, leukaemia inhibitory factor; Me_2SO, dimethyl sulfoxide

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growth factors and labeled with [methyl ^{3}H] thymidine for 28 h and processed for autoradiography. The percentage of cells that initiated DNA synthesis at a given time was determined as previously described [4,16].

2.3. Transfection

For stable transfections, 10^6 cells/ml were electroporated with pcDNA3.1 vector encoding *flag*-tagged wild type or dominant-negative-p38^{MAPK}[17]. After selection with G418 (400 μg/ml) and limiting dilution, multiple resistant clones were isolated and tested for p38^{MAPK} expression using anti-*flag* antibody. Cloned cells expressing the transgene were analyzed for cell proliferation and protein expression. The p38^{MAPK} constructs were a kind gift of Dr. JiaHuai Han, Scripps Research Institute, La Jolla, CA.

2.4. SDS–PAGE and immunoblotting

Protein extracts were prepared as in Sauane et al. [14]. Fifty micrograms of protein were separated on SDS–polyacrylamide gels and blotted onto nitrocellulose membranes. Thereafter, membranes were blocked and incubated overnight in the primary antibody diluted in 5% (w/v) non-fat milk in TBS-T. The primary antibodies used were: phospho-ERK$_{1\text{-}2}$ (#sc-7383), ERK$_2$ (#sc-154), p38^{MAPK} (#sc-535), cdk4 (#sc-260), cyclin D$_1$ (#sc-450) from Santa Cruz Biotechnology; phospho-p38^{MAPK} (#9211) from Cell Signaling; Retinoblastoma (pRb) (#14001A) from Pharmingen; *flag* (#F3165) from Sigma. Membranes were washed with TBS-T and incubated with peroxidase-conjugated secondary antibodies (Dako). The immunoblots were developed with the ECL detection reagent (Amershan). All the data shown are representative of three independent experiments.

2.5. Cyclin-dependent kinase assay

Cyclin D/cdk4-associated kinase activity was performed as described previously [18]. Briefly, quiescent and confluent cells were stimulated and harvested at the indicated times. Cyclin/cdk4 complex was immunoprecipitated with an anti-cdk4 antibody. Kinase activity was measured using 0.5 μg GST-pRb as the substrate and 10 μCi [γ^{32}P]ATP at 30 °C for 30 min. Reaction was stopped and analyzed by SDS–PAGE and autoradiography.

3. Results and discussion

ERK$_{1\text{-}2}$ and p38^{MAPK} signaling pathways are involved in a diverse array of cellular responses. While MEK/ERK$_{1\text{-}2}$ is a well-characterized signaling pathway activated by growth factors and involved in cell proliferation, there is less evidence linking p38^{MAPK} activation with cell proliferation. In order to gain insights about the early signaling mechanisms that mediate the PGF$_{2\alpha}$ mitogenic response in Swiss 3T3 cells, we performed a Western blot analysis using specific antibodies for the activated form of ERK$_{1\text{-}2}$ (phospho-Thr^{200}/Tyr^{204}). PGF$_{2\alpha}$ promoted a sustained ERK$_{1\text{-}2}$ activation, inducing a maximum increase at 5 min (Fig. 1A), remaining active for at least 8 h (data not shown). The same results were obtained when ERK$_{1\text{-}2}$ activation was determined by an immunoprecipitation in vitro kinase activity assay (data not shown). Treatment of Swiss 3T3 cells with U0126, a specific inhibitor of MEK$_1$ and thus ERK$_{1\text{-}2}$ activation [19], caused a concentration-dependent reduction in PGF$_{2\alpha}$-induced ERK$_{1\text{-}2}$ activation (Fig. 1B). In order to assess whether ERK$_{1\text{-}2}$ activation is required for the mitogenic effect of PGF$_{2\alpha}$, we treated the cells with U0126 before stimulation and DNA synthesis was measured. Interestingly, U0126 treatment strongly suppressed PGF$_{2\alpha}$-induction of DNA synthesis in a dose-dependent manner (Fig. 1C), whilst U0126 treatment did not significantly affect the mitogenic stimulus of fetal bovine serum (FBS) (Fig. 1C). This result suggested that the MEK$_1$/ERK$_{1\text{-}2}$ signaling pathway is critically involved in the PGF$_{2\alpha}$-proliferative response.

PGF$_{2\alpha}$-stimulation of DNA synthesis in Swiss 3T3 cells also requires PKC activation [14], and PKC activation has been shown to be one of the possible pathways leading to ERK$_{1\text{-}2}$

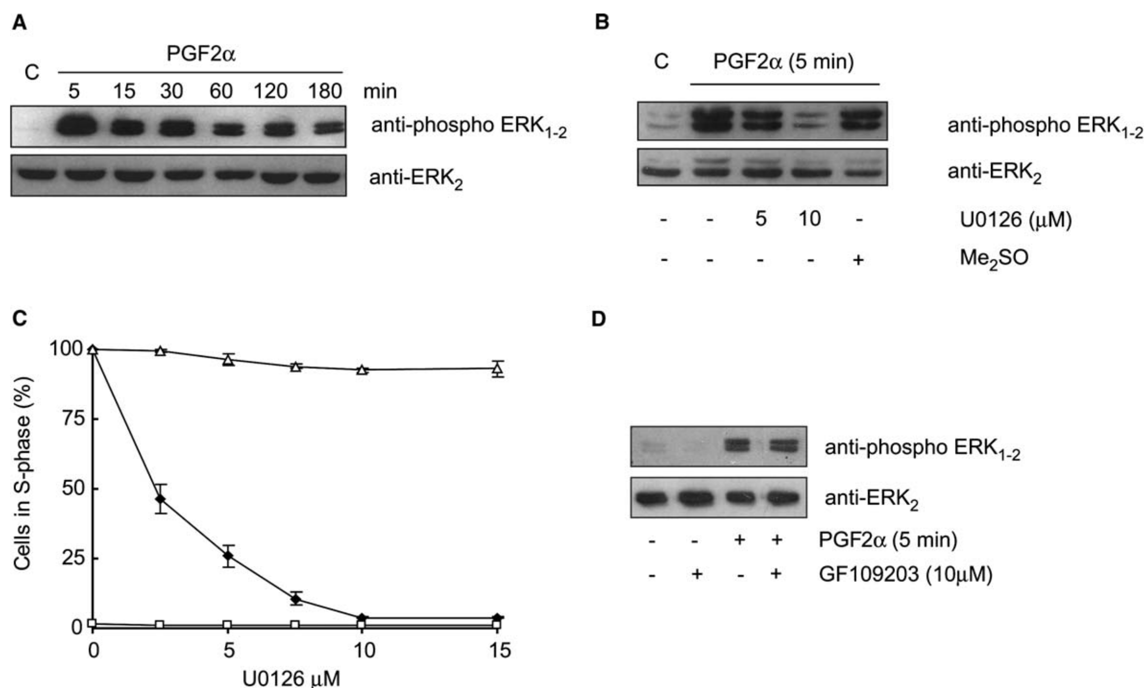

Fig. 1. U0126 blocked PGF$_{2\alpha}$-triggered ERK$_{1\text{-}2}$ activation and DNA synthesis. Quiescent cells were treated with PGF$_{2\alpha}$ (300 ng/ml) in the absence (A) or presence of U0126 or solvent control dimethyl sulfoxide (Me$_2$SO) (B) or GF109203 (D) at the indicated times. Equal amounts of proteins were analyzed by Western blot using phospho-ERK$_{1\text{-}2}$ (upper panel) or ERK$_2$ antibodies (lower panel). (C) To measure the effect of ERK$_{1\text{-}2}$ inhibition on PGF$_{2\alpha}$-stimulated DNA synthesis, quiescent cells were untreated (□), treated with PGF$_{2\alpha}$ (300 ng/ml; ◆) or FBS (10%; △) in the absence or presence of U0126 (0–15 μM). The percentage of S-phase cells was determined as described in Section 2. Results from one out of at least three independent experiments leading to the same conclusions are displayed.

activation [20]. Thus, to determine whether $PGF_{2\alpha}$-triggered ERK_{1-2} activation is a downstream event of PKC activation, we used a specific PKC inhibitor (GF109203X). Interestingly, $PGF_{2\alpha}$ was still able to promote ERK_{1-2} activation in cells pretreated with GF109203X (Fig. 1D), strongly suggesting that ERK_{1-2} activation is one of the early PKC-independent signals triggered by $PGF_{2\alpha}$ that are involved in the initiation of DNA synthesis.

To gather additional insights on $PGF_{2\alpha}$-triggered signals, we carried out a Western blot analysis using an antibody specific for the activated form of $p38^{MAPK}$ (phospho-Thr^{180}/Tyr^{182}). $PGF_{2\alpha}$ caused an increase in phospho-$p38^{MAPK}$ levels after 5 min of $PGF_{2\alpha}$ addition (Fig. 2A) reaching levels comparable to those obtained with osmotic stress induced by NaCl addition (Fig. 2A). To determine the role of this activation in the $PGF_{2\alpha}$ proliferative response, we treated the cells with the pyridinyl imidazole SB203580, a specific inhibitor of $p38^{MAPK}$. The ability of $PGF_{2\alpha}$ to induce DNA synthesis in Swiss 3T3 cells was blocked by addition of SB203580, causing maximal inhibition at 10 μM (Fig. 2B), whilst SB203580 treatment did not significantly affect the mitogenic stimulus of FBS or leukaemia inhibitory factor (LIF) (Fig. 2B). Further confirmation that $p38^{MAPK}$ is critically involved in $PGF_{2\alpha}$-induction of DNA synthesis, we generated stable cell lines expressing wild type or dominant negative $p38^{MAPK}$ (AF-$p38^{MAPK}$). Expression of phosphorylation-defective $p38^{MAPK}$ mutant with substitutions at Thr^{180} and Tyr^{182} by alanine and phenylalanine, respectively, has been shown to block $p38^{MAPK}$ activation in vivo [21]. As shown in Fig. 2C, the percentage of cells entering into the S-phase in response to $PGF_{2\alpha}$ were comparable between empty vector and wild type-$p38^{MAPK}$ transfected cells. In contrast, in AF-$p38^{MAPK}$transfected cells, $PGF_{2\alpha}$ failed to induce DNA synthesis (Fig. 2C). Cellular response to other mitogenic stimuli such as FBS or LIF was unaffected (Fig. 2C), consistent with a specific requirement of $p38^{MAPK}$ activation in $PGF_{2\alpha}$-induction of DNA synthesis.

We have previously reported that $PGF_{2\alpha}$ raised cyclin D_1 protein levels at 6–9 h, reaching a plateau value after 12–15 h and these levels remained relatively high for up to 21 h [14]. There is evidence that ERK_{1-2} activation raises cyclin D_1 levels, whilst $p38^{MAPK}$ activation causes the downregulation of cyclin D_1 [22]. However, our results shown that both pathways are required for the $PGF_{2\alpha}$ induction of DNA synthesis. Thus, G_1-cyclins expression was determined in $PGF_{2\alpha}$ stimulated cells in the presence of MEK_1 and $p38^{MAPK}$ inhibitors. Treatment of cells with U0126 caused a concentration-dependent reduction in $PGF_{2\alpha}$-triggered increases of cyclin D_1 levels (Fig. 3A). In contrast, cyclin D_1 levels were not significantly affected in cells treated with SB203580 (Fig. 3A). As we have previously shown, cdk4 protein levels are constitutive and are not affected by any of these treatments (Fig. 3A). Consistent with the results obtained with SB203580, $PGF_{2\alpha}$-triggered increases of cyclin D_1 expression were not affected in cells stable expressing *flag*-tagged wild type or AF-$p38^{MAPK}$ proteins (Fig. 3B), further demonstrating that $p38^{MAPK}$ activation is not involved in cyclin D_1 expression. We also evaluated the effect of MAPK inhibitors on cyclin D/cdk4 associated kinase activity. While U0126 caused a reduction in cyclin D/cdk4 kinase activity after $PGF_{2\alpha}$ stimulation (Fig. 3C), it was not significantly affected in SB203580-treated cells (Fig. 3C). These results show that the inhibitory effect of SB203580 on S-phase entry is neither due to a regulation of cyclin D_1 protein levels nor by regulating the formation and activation of cyclin D/cdk4 kinase complex.

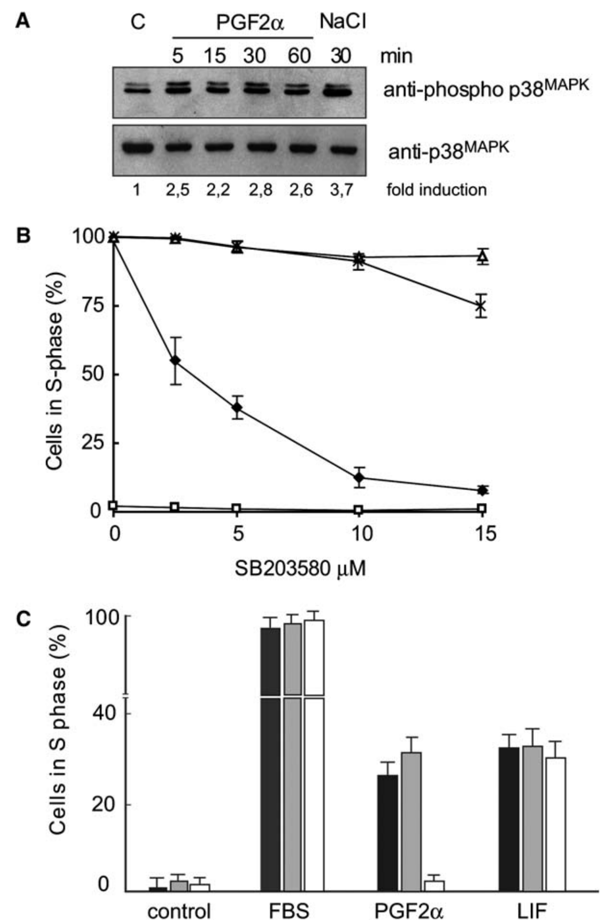

Fig. 2. $p38^{MAPK}$ activation is required for $PGF_{2\alpha}$-triggered DNA synthesis. (A) Cells were treated with $PGF_{2\alpha}$ (300 ng/ml) for 5–60 min or treated with NaCl (500 mM) for 30 min. Equal amounts of protein were analyzed by Western blot using phospho-$p38^{MAPK}$ (upper panel) or $p38^{MAPK}$ antibodies (lower panel). Band intensity was quantified; numbers indicate the ratio of phospho-p38/$p38^{MAPK}$ expressed as fold induction respect to untreated cells. Results from one out of at least three independent experiments leading to the same conclusions are displayed. (B) To determine the effect of $p38^{MAPK}$ inhibition on $PGF_{2\alpha}$-stimulated DNA synthesis, quiescent cells were untreated (□), treated with $PGF_{2\alpha}$ (300 ng/ml; ◆), FBS (10%; △) or LIF (100 ng/ml; ×) in the absence or presence of SB203580 (0–15 μM). The percentage of S-phase cells was determined as described in Section 2. (C) Quiescent cultures of stable transfected cells [empty vector (black), $p38^{MAPK}$ (grey) or AF-$p38^{MAPK}$ (white)] were untreated (control), treated with $PGF_{2\alpha}$ (300 ng/ml), FBS (10%) or LIF (100 ng/ml). The percentage of S-phase cells was determined as described in Section 2. The experiments were undertaken with five independent clones of stable transfected cells with the same results.

One event that precedes S-phase entry is hyper-phosphorylation of the pRb. To determine whether ERK_{1-2} and $p38^{MAPK}$ pathways converge prior to pRb phosphorylation, we analyzed the phosphorylation state of pRb by Western blot after $PGF_{2\alpha}$ stimulation of Swiss 3T3 cells. After $PGF_{2\alpha}$ or FBS addition, pRb appeared as a more slowly migrating band corresponding to hyperphospho-pRb protein (Fig. 4A). In contrast, this shift in pRb migration was absent when cells were treated with $PGF_{2\alpha}$ together with either U0126 or SB203580 (Fig. 4A),

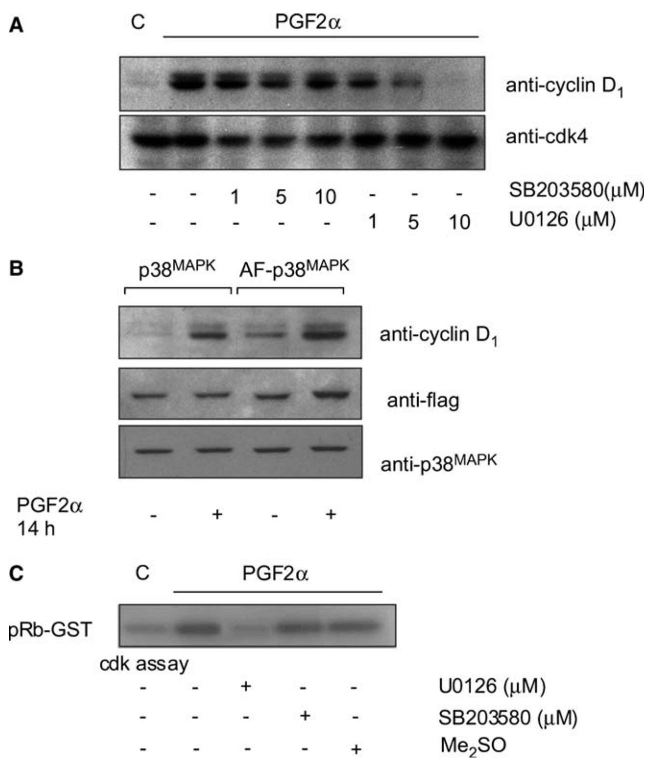

Fig. 3. $PGF_{2\alpha}$-triggered cyclin D1 expression and cyclin D/cdk4 activation require $ERK_{1\text{-}2}$ but not $p38^{MAPK}$activation. (A) Cells were untreated (c) or treated with $PGF_{2\alpha}$ in the absence or presence of U0126 or SB203580. Cell extracts were prepared and equal amounts of protein analyzed by Western blot using cyclin D_1 (upper panel) or cdk4 antibodies (lower panel). (B) *flag*-tagged wild type or AF-$p38^{MAPK}$ stable transfected cells were untreated or treated with $PGF_{2\alpha}$. Equal amounts of protein were analyzed by Western blot using cyclin D_1 (upper panel), *flag* (middle panel) or $p38^{MAPK}$ antibodies (lower panel). All these experiments were undertaken with five independent clones of stable transfected cells with the same results. (C) Cyclin D/cdk4-associated kinase activity was measured in vitro after $PGF_{2\alpha}$-stimulation of cells for 20 h in the absence or presence of U0126, SB203580 (10 μM) or equivalent amount of solvent (Me_2SO). Cyclin D/cdk4 complexes were immunoprecipitated and kinase activity measured using pRb-GST as substrate. Kinase reactions were analyzed by SDS–PAGE and autoradiography. Results from one out of at least three independent experiments leading to the same conclusions are displayed.

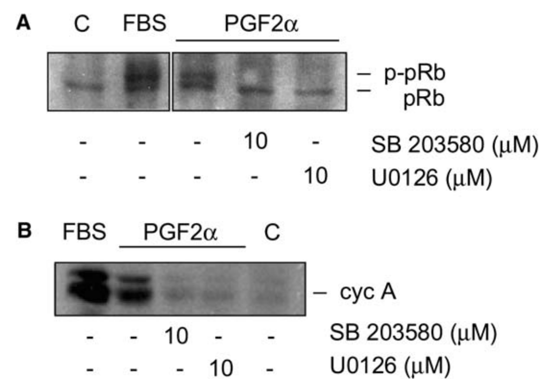

Fig. 4. Involvement of $ERK_{1\text{-}2}$ and $p38^{MAPK}$ on pRb phosphorylation and cyclin A expression. Cells were untreated (c), treated with FBS (10%) or treated with $PGF_{2\alpha}$ (300 ng/ml) for 25 h in the presence of U0126 and SB203580 (10 μM). Equal amounts of protein analyzed by Western blot using (A) a specific pRb antibody or (B) a specific cyclin A antibody. p-pRb; phosphorylated-pRb. Results from one out of at least three independent experiments leading to the same conclusions are displayed.

strongly suggesting that those events triggered by both signaling pathways are upstream of pRb. Similar results were obtained when cyclin A expression was analyzed (Fig. 4B); cyclin A is a transcriptional target of E2F and therefore downstream of pRb. These results further confirmed that both signaling pathways converge at a point prior to pRb. The results obtained using U0126 are as anticipated, since the inhibition of $ERK_{1\text{-}2}$ activation blocked cyclin D_1 expression and cyclin D/cdk4 activation. However, the results obtained with SB203580 suggest a role for $p38^{MAPK}$ activation in another process, different from activation of the cyclin D/cdk4 complex that is upstream of pRb inhibition.

In summary, we have shown using a combination of biochemical approaches and interfering mutants that both $ERK_{1\text{-}2}$ and $p38^{MAPK}$ activation are essential for $PGF_{2\alpha}$-stimulated S-phase entry. $PGF_{2\alpha}$ promoted $ERK_{1\text{-}2}$ activation by a PKC-independent pathway, suggesting that $ERK_{1\text{-}2}$ is one of the early PKC-independent signaling events involved in $PGF_{2\alpha}$-triggered DNA synthesis. We have also shown that both signaling pathways converge at a point prior to pRb phosphorylation. $ERK_{1\text{-}2}$ activation is required to induce cyclin D_1 expression, consistent with previous studies linking sustained ERK activation with cyclin D_1 expression and cellular proliferation [23]. The finding that $PGF_{2\alpha}$ triggers $ERK_{1\text{-}2}$ activation via a PKC-independent event are also supported by these results, since PKC inhibition blocked $PGF_{2\alpha}$'s mitogenic response, without affecting cyclin D_1 mRNA/protein expression [14]. $p38^{MAPK}$ activation is not related to cyclin D_1

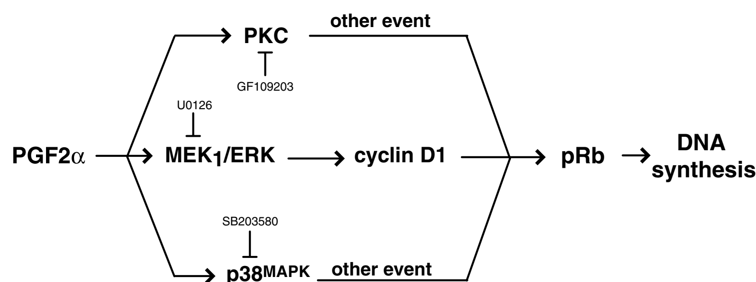

Fig. 5. Summary of $PGF_{2\alpha}$ triggered signals: $PGF_{2\alpha}$ induces PKC, $ERK_{1\text{-}2}$ and p38MAPK activation. Inhibition of any of these signaling pathways completely blocks $PGF_{2\alpha}$ induction of DNA synthesis. $ERK_{1\text{-}2}$ activation is required to induce cyclin D1 expression, while p38MAPK activation is involved in eliciting another process, strongly suggesting that the concerted action of cyclin D1 gene expression and other events are required to induce complete phosphorylation of pRb and S-phase entry in response to $PGF_{2\alpha}$.

expression nor cyclin D/cdk4 kinase activation suggesting a role for p38^{MAPK} in another process, different from cyclin D/cdk4 activation, that is required to induce DNA synthesis (Fig. 5). The involvement of other cyclin/cdk complexes (e.g. cyclin E/cdk2) is the subject of current active investigation.

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