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Leukemia Inhibitory Factor Induces DNA Synthesis in Swiss Mouse 3T3 Cells Independently of Cyclin D_1 Expression through a Mechanism Involving MEK/ERK1/2 Activation*

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Leukemia inhibitory factor (LIF) and oncostatin M (OSM) induce DNA synthesis in Swiss 3T3 cells through common signaling mechanism(s), whereas other related cytokines such as interleukin-6 and ciliary neurotrophic factor do not cause this response. Induction of DNA replication by LIF or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) occurs, in part, through different signaling events. LIF and OSM specifically trigger STAT1 cytoplasmic to nuclear translocation, whereas $PGF_{2\alpha}$ fails to do so. However, LIF and $PGF_{2\alpha}$ can trigger increases in ERK1/2 activity, which are required for their mitogenic responses because U0126, a MEK1/2 inhibitor, prevents both ERK1/2 activation and induction of DNA synthesis by LIF or $PGF_{2\alpha}$ treatment. $PGF_{2\alpha}$ induces cyclin D expression and full phosphorylation of retinoblastoma protein. In contrast, LIF fails to promote increases in cyclin D mRNA/protein levels; consequently, LIF induces DNA synthesis without promoting full phosphorylation of retinoblastoma protein (Rb). However, both LIF and $PGF_{2\alpha}$ increase cyclin E expression. Furthermore, LIF mitogenic action does not involve protein kinase C (PKC) activation, because a PKC inhibitor does not block this effect. In contrast, PKC activity is required for $PGF_{2\alpha}$ mitogenic action. More importantly, the synergistic effect between LIF and $PGF_{2\alpha}$ to promote S phase entry is independent of PKC activation. These results show fundamental differences between LIF- and $PGF_{2\alpha}$-dependent mechanism(s) that induce cellular entry into S phase. These findings are critical in understanding how LIF and other related cytokine-regulated events participate in normal cell cycle control and may also provide clues to unravel crucial processes underlying cancerous cell division.

Leukemia inhibitory factor (LIF)[5] belongs to a closely related group of cytokines, which includes oncostatin M (OSM), ciliary neurotrophic factor (CNTF), interleukin 6 (IL-6), and cardiotrophin-1 (1–4). Depending on the cell type, LIF promotes cellular proliferation or differentiation, *e.g.* embryonic stem cell growth (5, 6), mammalian embryo implantation (2, 4), neuronal differentiation (7, 8), enhancing survival of peripheral neurons (7) and oligodendrocytes (10), promoting bone formation (11), and myoblast proliferation (12–14). LIF is also implicated in a variety of pathophysiological processes (15–19). Cellular responses to LIF as well as to other cytokines are initiated via heterodimerization of two members of the cytokine receptor family (8, 20, 21). The resultant signal transduction process involves activation of cytoplasmic Janus kinases (8, 20, 21), which in turn promote tyrosine phosphorylation of the signal transducers and activators of transcription (STATs), thereby enabling them to translocate to the nucleus and initiate gene transcription of LIF-responsive genes (22). LIF can also trigger alternative signaling processes to those causing STAT activation (23). These include activation of the mitogen-activated protein kinase (MAPK) cascade, including the mitogen-activated protein kinase kinase (MAPKK or MEK), the MAPK isoenzymes (ERK1 and ERK2), and activation of S6 protein kinase (8).

We have shown previously that Swiss 3T3 cells are equally responsive to both sets of growth factors; LIF and $PGF_{2\alpha}$ are thus equally effective at inducing DNA synthesis (25). The generality of the difference in signaling events triggered by both cytokines and growth factors in different cell systems is well established; cytokines trigger activation of Janus kinases that promote phosphorylation of STATs (8, 20–22), and growth factors triggered mitogen-induced Raf/MEK/ERK signaling pathway leading to overexpression of cyclin D (26–29, 42–45). However, because these mitogens have been tested in different cellular systems, it is unknown whether this difference is a function of the cell type or is a fundamental difference in the delivery of the transducing signal *per se*. Thus, we have systematically studied LIF-, OSM-, and $PGF_{2\alpha}$-dependent mechanisms of control of S phase entry into the Swiss 3T3 cell system. We have shown previously that LIF-triggered signals differ from those triggered by classical mitogens, such as $PGF_{2\alpha}$, bombesin, or epidermal growth factor (30–32) in Swiss 3T3 cells. LIF and OSM trigger initiation of DNA synthesis without the requirement for mevalonic acid synthesis because inhibition of the hydroxymethylglutaryl-CoA reductase by lovostatin does not block LIF- or OSM-induced DNA replication and cell multiplication (32). Indeed, because LIF triggers cellular entry into the S phase via a PKC-independent signaling mechanism, it becomes relevant to investigate which activation cascade (MAPK and/or JAK/STAT) is involved in the onset of DNA replication and cell

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[5] The abbreviations used are: LIF, leukemia inhibitory factor; OSM, oncostatin M; IL, interleukin; CNTF, ciliary neurotrophic factor; MOPS, 4-morpholinepropanesulfonic acid; STAT, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; PG, prostaglandin; Rb, retinoblastoma protein; pRb, phosphorylated retinoblastoma protein; PKC, protein kinase C; JAK, Janus kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PBS, phosphate-buffered saline; MBP, myelin basic protein; FBS, fetal bovine serum; TBS, Tris-buffered saline.

division. Here we show that LIF and OSM stimulate the initiation of DNA synthesis and cell division through a common signaling mechanism that involves MEK/ERK activation as well as STAT1 cytoplasmic-nuclear translocation. Signals triggered by LIF and OSM are independent of PKC activation. On the other hand, $PGF_{2\alpha}$ triggers activation of MEK/ERK but fails to activate STAT1 cytoplasmic-nuclear translocation. The different signaling pathways involved in the mitogenic response to cytokines and growth factors cause a major effect on G_1 cyclin expression. $PGF_{2\alpha}$, as the majority of classical growth factors, induces the expression of cyclin Ds as an important step in the G_0 to S phase transition. However, LIF-stimulated S phase entry occurs without changes in the levels of cyclin Ds but with increases in cyclin E expression. These findings are relevant for understanding how LIF, in particular, and cytokines, in general, regulate the events involved in controlling the normal cell cycle. These studies are also important for unraveling critical signaling events capable of underlying unrestricted cancerous cell division.

MATERIALS AND METHODS

Chemicals—The majority of the materials was purchased from Sigma unless otherwise indicated. The mouse recombinant carrier-free LIF, OSM, CNTF, and IL-6 were purchased from R & D (Minneapolis, MN). $PGF_{2\alpha}$ was the generous gift from Dr. M. Torkelston, Upjohn (Kalamazoo, MI), and the GF109203X was kindly provided by GlaxoSmithKline. U0126 was purchased from Calbiochem. [*methyl*-^{3}H]Thymidine was purchased from PerkinElmer Life Sciences.

Cell Culture—Swiss mouse 3T3 cells were grown in Dulbecco's modified Eagle's medium containing 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 10% (v/v) fetal calf serum. Subconfluent cultures were grown in 100-mm dishes at 37 °C equilibrated with 10% (v/v) CO_2.

Initiation of DNA Synthesis Assay—Cells were seeded at 1.5×10^5 in 35-mm dishes in 2 ml of Dulbecco's modified Eagle's medium supplemented with low molecular weight nutrients (DEMS), 1% (v/v) newborn calf serum, and 6% (v/v) fetal calf serum. After 3 days of incubation, the medium was changed to a similar fresh medium, and the cells were further incubated for 3–4 days to allow them to become confluent and quiescent. Cytokines and growth factors were directly added to the conditioned medium. Cells were then labeled with [*methyl*-^{3}H]thymidine for 28 h before being processed for autoradiography. The percentage of cells that initiated DNA synthesis at a given time was determined as described previously (25, 33).

Cell Number—Cells were plated at 1.5×10^5 in 60-mm dishes in 5 ml of medium, for the determination of the initiation of DNA synthesis. When cells became quiescent, but were still subconfluent, the corresponding cytokines or prostaglandins were added to the culture medium, and the cell number was determined 60 h later. Cells were detached from the dish using 5 mM EDTA, 0.05% (v/v) trypsin for 5 min at 37 °C and counted in a Coulter counter (34).

MAPK Assay—MAPK activity assays were performed on subconfluent Swiss 3T3 cells by immunoprecipitation of total endogenous ERK. Cells were maintained for 16 h in serum-free medium and then treated with agents as indicated in the figure legends, washed with cold PBS, and lysed at 4 °C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1% (v/v) Triton X-100, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. ERK was immunoprecipitated from the cleared lysates by incubation with the specific antibody (sc-154 from Santa Cruz Biotechnology) for 1 h at 4 °C. Immunocomplexes were recovered with the aid of Gamma-bind-Sepharose Beads (Amersham Biosciences) and washed three times with PBS containing 1% (v/v) Nonidet P-40 and 2 mM Na_3VO_4, once with 100 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and once in kinase reaction buffer (12.5 mM MOPS (pH 7.5), 12.5 mM 3-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate). The ERK-2 activity present in the immunoprecipitates was determined by resuspension in 30 μl of kinase reaction buffer containing 10 μCi of [γ-^{32}P]ATP per reaction and 20 μM of unlabeled ATP, using 20 μg of myelin basic protein (MBP) as a substrate, as described previously (35). After 20 min at 30 °C, reactions were terminated by addition of 10 μl of 5× Laemmli buffer. Samples were heated 5 min at 95 °C and analyzed by SDS-gel electrophoresis on 12% (w/v) polyacrylamide gels. Autoradiography was performed with the aid of an intensifying screen. Parallel immunoprecipitates were processed for Western blot analysis using the same antiserum as described previously (35).

Indirect Immunofluorescence—It was performed on sparse quiescent Swiss 3T3 cells, adhering to coverslips. After stimulation, cells were fixed and permeabilized with 3.7% (v/v) formaldehyde in PBS plus 0.2%(v/v) saponin for 10 min at room temperature. Cells were then blocked for 30 min with 10% (v/v) heat-inactivated goat serum and then incubated overnight at 4 °C with 20 μg/ml of the primary antibody diluted in PBS, 0.1%(v/v) saponin. The primaries antibodies used were as follows: STAT1 antibody (G16920), STAT3 antibody (S21320), and STAT5 antibody (S21520) from BD Transduction Laboratories. The coverslips were washed three times with PBS and further incubated for 1 h with fluorescein isothiocyanate-conjugated goat anti-mouse IgGs (Sigma) diluted 1/50 in PBS. Finally the cells were washed three times with PBS and mounted with 1,4-diazabicyclo[2.2.3]octane solution (Sigma). Images were obtained on a BX-60 Olympus fluorescent microscope. The percentage of cells in which STAT1 translocated to the nucleus upon LIF or LIF plus Na_3VO_4 treatment was determined in three independent experiments by counting the number of nuclear and non-nuclear stained cells in at least five fields, each containing an average of at least 150 cells, at different time points.

SDS-PAGE and Immunoblotting—Proteins were separated on 12% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose filters. Transfers were blocked overnight at 4 °C with 5% (v/v) nonfat milk in TBS, 0.1% (v/v) Tween 20 and then incubated for 1 h at room temperature in the primary antibody diluted in 5% (v/v) nonfat milk in TBS, 0.1%(v/v) Tween 20. The primary antibodies used were as follows: phosphospecific p42/p44^{MAPK} antibody (sc-7383; diluted 1/1000), p42/p44^{MAPK} antibody (sc-154; 1/1000), cyclin D_1 antibody (sc-450; 1/1000), cyclin D_2 antibody (sc-593; 1/1000), cyclin D_3 antibody (sc-6283; 1/1000), cyclin E antibody (sc-481; 1/1000), CDK4 antibody (sc-260; 1/1000), and CDK6 antibody (sc-177; 1/1000) from Santa Cruz Biotechnology; cyclin A antibody (C4710) from Sigma. The transfers were rinsed with TBS, 0.1% (v/v) Tween 20 and incubated for 1 h at room temperature in horseradish peroxidase-conjugated pig anti-rabbit or rabbit anti-mouse serum (Dako) diluted 1/5000 in 5% (v/v) nonfat milk in TBS, 0.1% (v/v) Tween 20. The immunoblots were developed with the ECL detection reagent (Amersham Biosciences).

Northern Blot—Cells were plated at 3.0×10^5 in 100-mm dishes similar to the assay for DNA synthesis. Under these conditions, cultures became confluent and quiescent at a saturating density of 3×10^6 cells. In experiments where the levels of cyclin D_1 mRNA were determined after various times of addition of growth factors, total RNA was prepared from cells by extracting them with Trizol reagent. Samples (15 μg) were subjected to 1% (v/v) MOPS/formaldehyde agarose-gel electrophoresis and blotted onto nylon membranes. The membranes were hybridized with ^{32}P-labeled cDNA probes for cyclin D_1 and 18 S RNA

with a Promega kit, washed, and exposed to x-ray film (36). For cyclin D_1 the 1.3-kbp EcoRI fragment of pcBZ04.1 was used. Cyclin D_1 and 18 S RNA cDNA probes were generously provided by Drs. C. D. Sherr (St. Jude's Hospital, Memphis, TN) and A. R. Kornblihtt (Physiology and Molecular Biology Laboratory, School of Sciences, University of Buenos Aires, Argentina), respectively.

RESULTS

LIF and OSM Induce DNA Synthesis through Common Signaling Mechanism(s)—To investigate whether LIF-triggered signals that induce cell proliferation can be shared by other related cytokines, such as OSM, CNTF, and IL-6, we studied their ability to induce cellular entry into S phase in resting Swiss 3T3 cells. Both LIF and OSM, added at either subsaturating (10 ng/ml) or saturating (100 ng/ml) concentrations, stimulated the initiation of DNA synthesis inducing 8 and 29% of cells to enter into S phase after 28 h, respectively (Fig. 1), as shown previously (32). When both LIF and OSM were added together, they caused only an additive effect on the percentage of cells that entered into S phase at any concentration tested (Fig. 1). In contrast, treatment of Swiss 3T3 cells with IL-6 or CNTF did not induce cellular entry into the S phase (Fig. 1). Taken together, these results suggest that LIF and OSM may share common signaling pathway(s) to induce the entry into S phase.

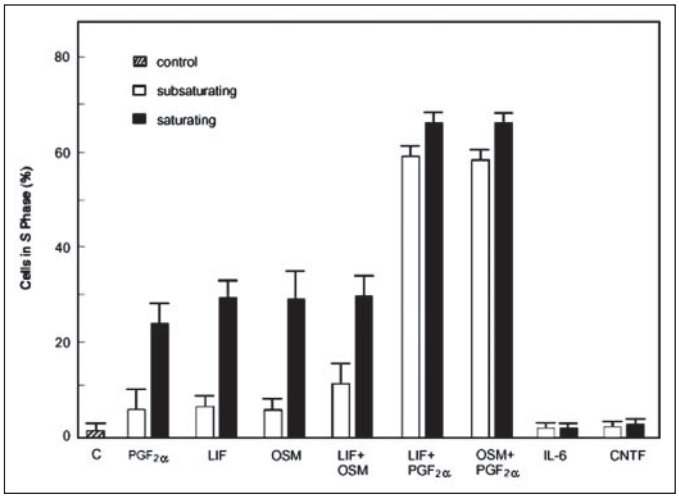

FIGURE 1. **Induction of DNA synthesis by LIF and OSM.** To measure cellular entry into S phase, quiescent Swiss 3T3 cells were treated with each cytokine and growth factor at saturating or subsaturating concentrations and then labeled with [*methyl*-^{3}H]thymidine for 28 h. Radioactive label incorporation was analyzed by autoradiography (see under "Materials and Methods"). Additions were as follows: LIF (10 ng/ml), OSM (10 ng/ml), CNTF (10 ng/ml), IL-6 (10 ng/ml), $PGF_{2\alpha}$ (30 ng/ml) at subsaturating concentrations, and LIF (100 ng/ml), OSM (100 ng/ml), CNTF (100 ng/ml), IL-6 (100 ng/ml), and $PGF_{2\alpha}$ (300 ng/ml) at saturating concentrations. Results represent the mean ± S.E. of four independent experiments.

To examine whether LIF- or OSM-triggered signals differ from those elicited by $PGF_{2\alpha}$, we analyzed the ability of $PGF_{2\alpha}$ to enhance LIF- or OSM-dependent induction to S phase entry. $PGF_{2\alpha}$ treatment stimulated the initiation of DNA synthesis in Swiss 3T3 cells by inducing 7 and 25% of cells to enter into S phase after 28 h (Fig. 1), similarly to the effect observed with LIF or OSM. Most interestingly, when $PGF_{2\alpha}$ and LIF were added together at either subsaturating or saturating concentrations, the stimulatory effect of LIF or $PGF_{2\alpha}$ in inducing DNA synthesis was potentiated by raising the fraction of cells that entered into S phase to 57 and 67%, respectively (Fig. 1). Similarly, the stimulatory effect of OSM was potentiated by $PGF_{2\alpha}$ (Fig. 1). These results suggest that LIF or OSM in combination with $PGF_{2\alpha}$ exhibited a synergistic effect by increasing the percentage of cells entering S phase. Furthermore, we hypothesized that the synergistic effect observed between LIF or OSM with $PGF_{2\alpha}$ to induce both DNA synthesis and cell division may be due, at least in part, to the activation of different signaling pathways promoted by LIF and OSM with respect to those activated by $PGF_{2\alpha}$.

LIF and $PGF_{2\alpha}$ Activate MAPK Signaling Pathway with a Different Kinetic Pattern—To ascertain whether the mitogenic effect of LIF and $PGF_{2\alpha}$ on Swiss 3T3 cells involved differential activation of a well characterized MAPK cascade (37), we examined the capacity of LIF and $PGF_{2\alpha}$ to activate ERK. Treatment of Swiss 3T3 cells with LIF or $PGF_{2\alpha}$ resulted in different kinetic patterns of ERK activation. LIF promoted a maximum increase of ERK activity only at 12 min (Fig. 2*B*), whereas $PGF_{2\alpha}$ caused a maximum increase in ERK activity within 4 min (Fig. 2*A*). There was no change in the levels of p44/p42 MAPK throughout this period (Fig. 2, *A* and *B*).

Because both LIF and $PGF_{2\alpha}$ promote the activation of ERK1/2, we investigated whether LIF- or $PGF_{2\alpha}$-triggered activation of ERK is required for their mitogenic effect. Treatment of cells with U0126, a widely used MEK inhibitor (38), for 1 h before addition of growth factors caused a significant and progressive reduction in either LIF- or $PGF_{2\alpha}$-induced ERK phosphorylation without affecting the overall levels of this protein (Fig. 3*A*). Indeed, U0126 at 10 μM inhibited ERK activity by 90% in both $PGF_{2\alpha}$- and LIF-stimulated cells (Fig. 3*B*). Moreover, addition of U0126 from 0.5 to 5 μM to cells prior to LIF or $PGF_{2\alpha}$ treatment resulted in a progressive inhibition of DNA replication (Fig. 3*C*). However, although U0126 at 1 μM effectively blocked $PGF_{2\alpha}$-stimulated DNA synthesis by 90%, a higher concentration of 3 μM was required to cause a similar effect with LIF-stimulated cells. In contrast, U0126 did not

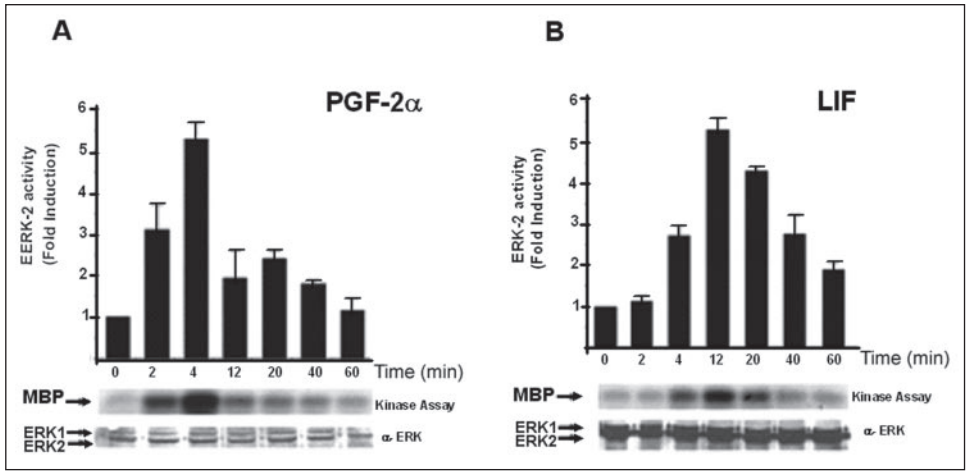

FIGURE 2. **LIF or $PGF_{2\alpha}$ triggered ERK activation with a different kinetic pattern.** Quiescent Swiss 3T3 cells were treated with $PGF_{2\alpha}$ (300 ng/ml) (*A*) or LIF (100 ng/ml) (*B*). Cells were lysed at time intervals from 0 to 60 min, and kinase activity was measured as indicated under "Materials and Methods." Data represent the mean ± S.E. of three independent experiments, expressed as fold increase in kinase activity with respect to untreated cells. *Upper panel,* densitometric analysis expressed as fold increase in kinase activity with respect to untreated cells; *middle panel,* ^{32}P-labeled MBP as product of the kinases reaction for one representative experiment; *lower panel,* equal amounts of ERK protein were immunoprecipitated as shown by Western blot analysis of the immunoprecipitated samples using anti-ERK antibodies.

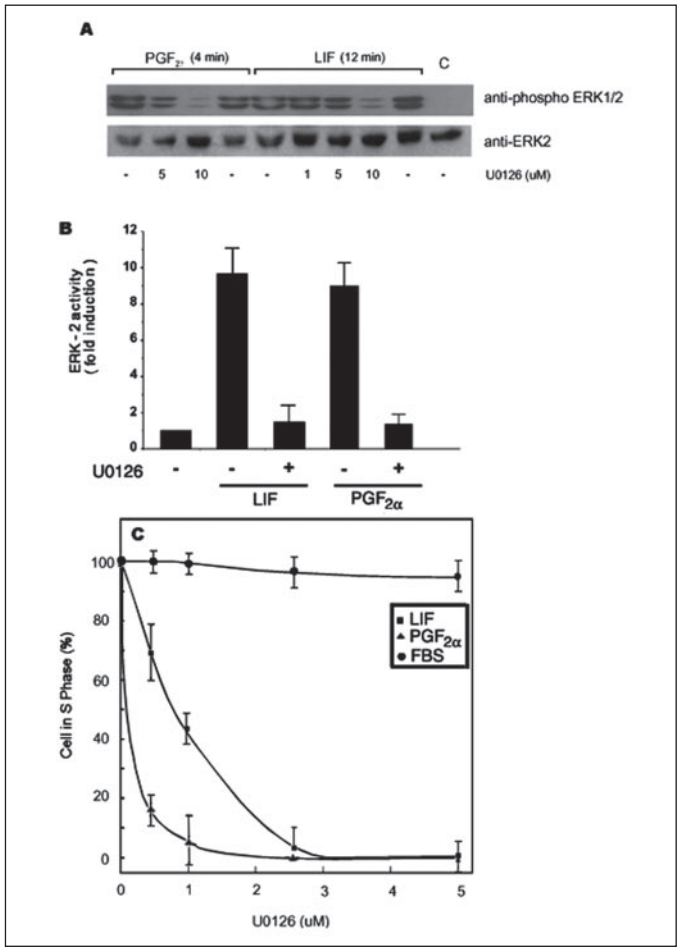

FIGURE 3. **ERK activation is required for LIF- or $PGF_{2\alpha}$-dependent DNA synthesis**. *A,* cells were treated with vehicle (*lane C*), LIF (100 ng/ml), or $PGF_{2\alpha}$ (300 ng/ml) for 12 and 4 min, respectively, in the absence or presence of U0126. Cells were lysed, and equal amounts of protein were analyzed by Western blot using a specific phospho-ERK1/2 antibody (*upper panel*) or a specific ERK2 antibody (*lower panel*). The results are representative of three independent experiments. *B,* cells were left untreated or treated with LIF (100 ng/ml) or $PGF_{2\alpha}$ (300 ng/ml) for 12 and 4 min, respectively, in the absence or presence of U0126 (10 μM). Endogenous ERK2 protein was immunoprecipitated from the cell extracts and kinase activity assayed using MBP as substrate. Data represent the mean ± S.E. of three independent experiments expressed as fold increase in kinase activity with respect to untreated cells. *C,* to measure the effect of ERK inhibition on LIF- or $PGF_{2\alpha}$-stimulated DNA synthesis, quiescent Swiss 3T3 fibroblasts were treated for 28 h with LIF (100 ng/ml), $PGF_{2\alpha}$ (300 ng/ml), and FBS (10%v/v) in the absence or presence of the indicated concentrations of U0126 and then labeled with [*methyl*-^{3}H]thymidine. U0126 was added 1 h prior to the growth factors. The percentage of cells that entered into S phase was determined by autoradiography, as described under "Materials and Methods." The results are presented as the percentage of labeled nuclei with respect to the growth factor alone and are representative of four independent experiments.

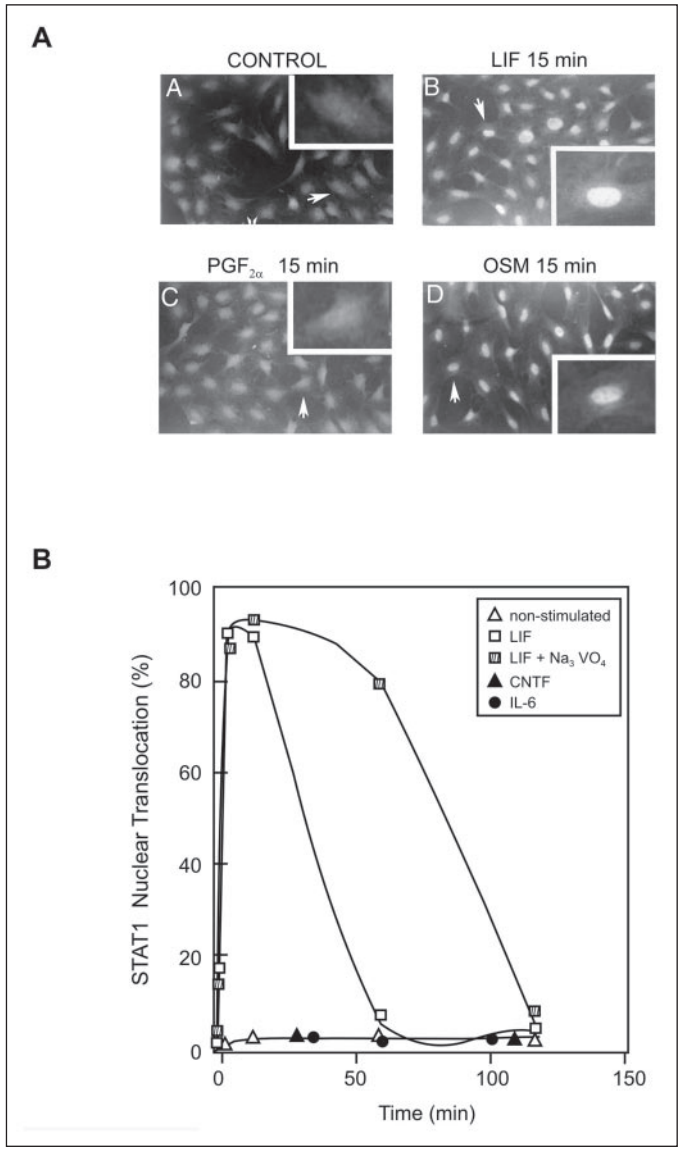

FIGURE 4. **LIF, but not $PGF_{2\alpha}$, promotes nuclear translocation of STAT1.** *A,* subconfluent Swiss 3T3 cells were treated with vehicle (*A*), LIF (100 ng/ml) (*B*), OSM (ng/ml) (*D*), or $PGF_{2\alpha}$ (300 ng/ml) (*C*) for 15 min. Cells were fixed, and STAT1 localization was visualized by indirect immunofluorescence with an anti-STAT1 monoclonal antibody as described under "Materials and Methods." *Insets* in panels show a higher magnification of a cell for better visualization. Percentage of cells with STAT1 nuclear staining is as follows: control, 10 ± 4%; LIF, 89 ± 5%; OSM, 85 ± 7%; and $PGF_{2\alpha}$, 15 ± 6%. *B,* kinetic pattern of STAT1 translocation was determined as follows. Swiss 3T3 cells were stimulated with LIF in the absence or presence of Na_3VO_4 (30 μM). This inhibitor was added 1 h prior to cell treatment. Cells were fixed, and the STAT1 localization was visualized by indirect immunofluorescence as described under "Materials and Methods." The percentage of STAT1-immunostained nuclei was determined at different times after cytokine addition. At least 750 cells were counted per time point. The results represent the means ± S.E. of three independent experiments.

affect DNA synthesis stimulated by fetal bovine serum (FBS). These results showed that ERK activation is required for both LIF- and $PGF_{2\alpha}$-triggered cellular entry into S phase, although with a different kinetic pattern.

Role of JAK/STAT Signaling Pathway in LIF/$PGF_{2\alpha}$-induced DNA Synthesis—To investigate whether LIF, OSM, and $PGF_{2\alpha}$ exert a differential stimulation of the JAK/STAT pathway, we examined their ability to cause cytoplasmic to nuclear translocation of the different STATs by indirect immunofluorescence upon treatment of subconfluent resting Swiss 3T3 cells. In control, nonstimulated cells, STAT1 was mainly diffusely distributed within the cytoplasm (Fig. 4*A*). LIF and OSM treatment promoted nuclear translocation of STAT1 as demonstrated by the nuclear immunostaining of STAT1 (Fig. 4, *panels A*, *B*, and *D*, respectively). In contrast, $PGF_{2\alpha}$ did not trigger STAT1 nuclear localization (Fig. 4*A*, *panel C*). Results similar to those found by immunofluorescence were obtained when the subcellular localization of STAT1 was determined by analyzing cytoplasmic and nuclear fractions of LIF-stimulated cells by Western blotting (data not shown). Furthermore, LIF, OSM, and $PGF_{2\alpha}$ could not promote translocation of STAT3 or STAT5 to the nucleus in the 3T3 cells (not shown). As positive controls, anti-STAT3 and STAT5 antibodies were able to detect STAT3 and STAT5 cytoplasmic to nuclear translocation in 3T3 L1 pre-adipocytes stimulated by different cytokines, growth factors, and hormones (data not shown) (39). No translocation was observed with either IL-6 or CNTF, which are not mitogenic for Swiss 3T3 cells (Fig. 4*B*).

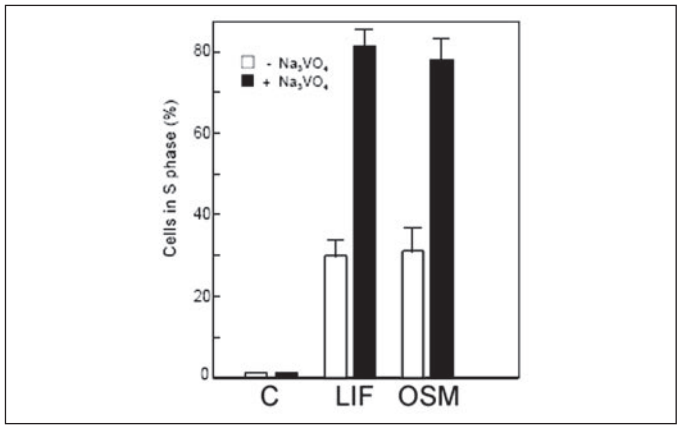

FIGURE 5. **Inhibition of tyrosine phosphatases enhances LIF induction of DNA synthesis.** To analyze the effect of tyrosine phosphatase inhibition on LIF stimulation of DNA synthesis, quiescent Swiss 3T3 cells were treated for 28 h with LIF (100 ng/ml) or OSM (100 ng/ml) in the absence or presence of 30 μM Na_3VO_4 and then labeled with [*methyl*-^{3}H]thymidine. *C*, control. The Na_3VO_4 was added 1 h before the cytokine treatment. The percentage of cells that entered into S phase was determined by autoradiography, as described under "Materials and Methods." Results represent the means ± S.E. of three independent experiments.

To ascertain whether regulation of tyrosine phosphatases is implicated in LIF-dependent nuclear localization of STAT1, Swiss 3T3 cells were incubated in the presence or absence of Na_3VO_4, a general inhibitor of tyrosine phosphatases (40, 41), prior to and during cytokine treatment. LIF-promoted cytoplasmic-nuclear translocation of STAT1 occurred as rapidly as 2 min after addition of LIF, and its nuclear localization attained a plateau at 10–15 min and declined to the basal level by 60 min (Fig. 4*B*). In the presence of Na_3VO_4, LIF also promoted the rapid translocation of STAT1 to the nucleus. However, in the presence of Na_3VO_4, STAT1 remains in the nucleus after 60 min of LIF treatment (Fig. 4*B*). This observation is consistent with findings that showed that a phosphatase inhibitor could prolong the activation of STAT1 and thus its nuclear retention (42). Indeed, Na_3VO_4 also markedly enhanced LIF-induced cellular entry into S phase, raising the percentage of cells undergoing DNA synthesis over 2.5-fold (Fig. 5). Na_3VO_4 also enhanced OSM- and $PGF_{2\alpha}$-dependent cellular entry into the S phase (Fig. 5). These experiments indicated that LIF-dependent activation of STAT1 and initiation of DNA replication might both involve tyrosine kinase activation. In contrast, the $PGF_{2\alpha}$-dependent mitogenic effect appears not to require STATs activation.

Effect of LIF and $PGF_{2\alpha}$ on G_1 Cyclin Expression and pRb Phosphorylation—To determine whether differences between LIF/OSM and PGF2α signaling pathways have major consequences on expression of key G_1 cyclins and their effector kinases involved in executing the G_1/S transition, the expression of cyclins was assessed at different times upon treatment of quiescent 3T3 cells with LIF or $PGF_{2\alpha}$. Fig. 6 shows that $PGF_{2\alpha}$ raised cyclin D_1 protein levels within 9 h, reaching a plateau after 12–15 h, and these levels remained relatively high for up to 21 h (Fig. 6*A*). $PGF_{2\alpha}$ also raised cyclin D_2 protein levels at later times (within 15–21 h of treatment) but failed to increase cyclin D_3 protein levels (Fig. 6*A*). In contrast, LIF as well as OSM failed to increase cyclin D_1 protein and mRNA levels (Fig. 6, *A* and *B*), as well as failing to raise cyclin D_2 or cyclin D_3 protein levels (Fig. 6*A*). IL-6 and CNTF were also unable to induce cyclin D_1 mRNA levels (Fig. 6*B*). The levels of the corresponding partner CDK4/6 kinases (43) did not show any increase upon LIF or $PGF_{2\alpha}$ treatments (Fig. 6*A*). However, addition of LIF induced an increase in cyclin E and cyclin A protein levels after 14 and 28 h to levels comparable with that induced by 10% FBS, at least for cyclin E (Fig. 6*C*).

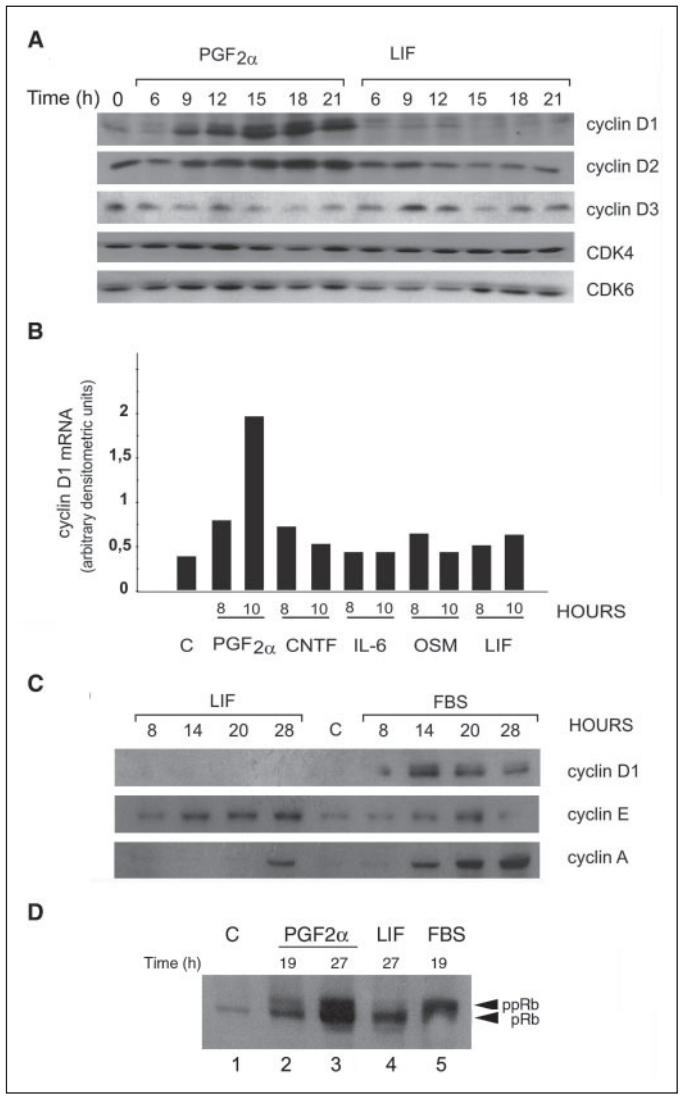

FIGURE 6. **The mitogenic effect of LIF is independent of cyclin D expression.** *A*, Swiss3T3 cells were treated with vehicle or saturating concentrations of LIF (100 ng/ml) or $PGF_{2\alpha}$ (300 ng/ml) for different periods of time. Cell extracts were prepared, and equal amounts of protein were analyzed by immunoblotting using specific antibodies for cyclin D_1, cyclin D_2, cyclin D_3, CDK4, and CDK6 (see "Materials and Methods"). Data are representative of three independent experiments. *B*, cyclin D_1 mRNA levels were determined at various times after cytokine or $PGF_{2\alpha}$ treatment. Swiss 3T3 cells were treated with vehicle or saturating concentrations of LIF (100 ng/ml), OSM (100 ng/ml), CNTF (100 ng/ml), IL-6 (100 ng/ml), or $PGF_{2\alpha}$ (300 ng/ml). After 8–10 h, total RNA was extracted from cells; Northern blot was performed as described under "Materials and Methods." Northern blot densitometric analysis was standardized to 18 S RNA. Similar results were obtained in three independent experiments. *C*, Swiss 3T3 cells were treated with vehicle, LIF, or FBS for various times. Cell extracts were prepared, analyzed by SDS-PAGE, and immunoblotted using specific antibodies against cyclin D_1, cyclin E, and cyclin A. Results are representative of three independent experiments. *D*, extracts from cells treated with vehicle (*lane C*), $PGF_{2\alpha}$, LIF, or FBS for the indicated periods of time were separated by SDS-PAGE and subjected to immunoblot analysis for pRb. *Arrows* indicate pRb with different levels of phosphorylation.

$PGF_{2\alpha}$ increases cyclin E and A protein levels similar to those for LIF (data not shown).

Cyclin D-CDK complexes were shown to phosphorylate the retinoblastoma tumor suppressor protein (pRb), leading to inactivation of pRb (44). It is well documented that inactivation of pRb results in release or derepression of the E2F transcription factors and drives cell entry into the S phase (44). To examine whether differences in cyclin D expression mediated by LIF and $PGF_{2\alpha}$ result in differences in pRb phosphorylation, quiescent Swiss 3T3 cells were induced to enter S phase, and phosphorylation of pRb was assessed by immunoblotting. As shown in Fig.

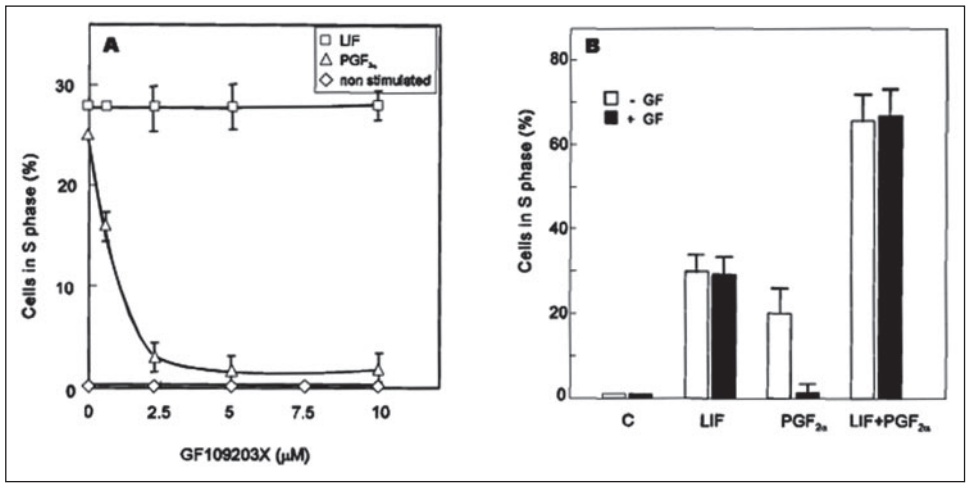

FIGURE 7. **The synergistic effect between LIF and $PGF_{2\alpha}$ to induce S phase entry is independent of PKC activation.** *A,* quiescent Swiss 3T3 cells were treated for 28 h with LIF or $PGF_{2\alpha}$ in the absence or presence of GF109203X added 1 h prior to the addition of growth factors and then labeled with [*methyl*-^{3}H]thymidine. The percentage of cells that entered into S phase was determined by autoradiography, as described under "Materials and Methods." *B,* quiescent Swiss 3T3 cells were treated for 28 h with vehicle, LIF, $PGF_{2\alpha}$, or LIF and $PGF_{2\alpha}$ in the absence or presence of GF109203X (10 μM) added 1 h prior to addition of growth factors. The percentage of cells that entered into S phase was determined by autoradiography. Results represent the mean ± S.E. of four independent experiments.

6*D,* LIF did not promote full phosphorylation of pRb (*lane 4 versus lane 1*). In contrast, $PGF_{2\alpha}$ induced hyperphosphorylation of pRb (Fig. 6*D, lanes 2* and *3 versus lane 1*), consistent with the induction of expression of cyclins Ds. Phosphorylation of pRB induced by $PGF_{2\alpha}$ is comparable with the level of pRb phosphorylation induced by FBS (Fig. 6*D, lane 5*) upon cell entry into S phase. In summary, LIF induces neither expression of cyclin Ds nor hyperphosphorylation of pRb, whereas $PGF_{2\alpha}$ promotes both the increase in the expression of cyclin Ds and the hyperphosphorylation of pRb to exert its mitogenic effect. Taken together, these results provide further evidence that LIF and $PGF_{2\alpha}$ may act through different signaling and molecular events to control the initiation of cellular entry into S phase.

The Synergistic Effect of LIF and $PGF_{2\alpha}$ to Induce S Phase Entry Is Independent of PKC Activation—We have shown previously that LIF and $PGF_{2\alpha}$ differ markedly in the requirement for PKC in stimulating DNA synthesis (25). LIF triggers cellular entry into S phase via a PKC-independent signaling mechanism, whereas $PGF_{2\alpha}$ requires the activation of the PKC signaling pathway (25). Therefore, we examined whether $PGF_{2\alpha}$-dependent activation of PKC plays a role in the synergistic effect observed between LIF and $PGF_{2\alpha}$ in the induction of DNA synthesis in Swiss 3T3 cells. We tested the effect of increasing concentrations of GF109203X, a specific inhibitor of PKC, on the ability of LIF and $PGF_{2\alpha}$ alone or in combination to trigger DNA replication. As shown in Fig. 7*A*, GF109203X progressively inhibited $PGF_{2\alpha}$-dependent DNA replication but completely failed to block LIF-dependent DNA replication. These results are consistent with our previous findings obtained with 12-*O*-tetradecanoylphorbol-13-acetate indicating that LIF and $PGF_{2\alpha}$ differed markedly in the requirement for PKC in stimulating DNA synthesis (25). Most interestingly, GF109203X did not prevent the synergistic effect between $PGF_{2\alpha}$ and LIF in increasing the percentage of cells that entered into S phase (Fig. 7*B*). These results suggest that the synergistic effect of LIF and $PGF_{2\alpha}$ to promote S phase entry is independent of PKC activation.

DISCUSSION

It has been shown previously that Swiss 3T3 cells are equally responsive to both sets of growth factors; LIF and $PGF_{2\alpha}$ are thus equally effective at inducing DNA synthesis (25). The generality of the difference in signaling events triggered by both cytokines and growth factors in different cell systems is well established; cytokines trigger activation of Janus kinases that promote phosphorylation of STATs (8, 23–25), and growth factors trigger the mitogen-induced Raf/MEK/ERK signaling pathway leading to overexpression of cyclin D (26–29, 42–45). However, because these mitogens have been tested in different cellular systems, it is unknown whether this difference is a function of the cell type or is a fundamental difference in the delivery of the transducing signal *per se*. In this study we show that LIF-triggered signaling mechanism(s) for inducing cellular entry into S phase are shared only by OSM and not by $PGF_{2\alpha}$ in the same quiescent Swiss mouse 3T3 cell system. Their differences are not due to subpopulations of 3T3 cells that are primarily responsive to LIF/OSM or to $PGF_{2\alpha}$, because repeated subcloning of 3T3 cells and addition of LIF/OSM or $PGF_{2\alpha}$ yield the same percentages of cells stimulated to synthesize DNA in the given time period.[6] Treatment of Swiss 3T3 cells with LIF or OSM together with $PGF_{2\alpha}$ mutually potentiated their ability to induce cellular DNA synthesis, whereas co-treatment of cells with LIF and OSM rendered no further increase. Experimentally cellular DNA synthesis is measured by the fraction of cells with [^{3}H]DNA in their nuclei after 28 h. A longer exposure of cells to [^{3}H]thymidine with one mitogen increases this fraction, eventually reaching almost 100% of the cell population (22, 25, 27, 28, 33, 34). The synergy observed between LIF and $PGF_{2\alpha}$ merely increases the rate of cellular entry into S phase but not the absolute fraction of cells that are responding. These observations suggest that LIF and OSM trigger common signaling pathways that may differ from those activated by $PGF_{2\alpha}$ for the induction of DNA synthesis. The importance of the study therefore lies in the uncovering of the biochemical differences in the signal transduction pathways of these two groups of mitogens in the same cell system.

ERK1/2 are components of the well known MAPK signaling cascade activated by mitogens and are thus involved in controlling cell proliferation (37). Here we show that both LIF or $PGF_{2\alpha}$ by themselves can promote ERK activation. However, LIF and $PGF_{2\alpha}$ differ in their timing of MAPK activation. Although $PGF_{2\alpha}$ induced a maximum at 4 min, LIF did so only after 12 min after addition, a result that suggests that LIF and $PGF_{2\alpha}$ cause ERK activation via two separate upstream signaling events. U0126, a highly specific MEK inhibitor, blocked both LIF- and $PGF_{2\alpha}$-triggered MAPK activation and their mitogenic responses, strongly suggesting that MAPK activation is required for the initiation of both LIF and $PGF_{2\alpha}$-dependent DNA synthesis. However, how LIF increases ERK activity and the consequent stimulation of DNA synthesis in these cells is still unknown. MAPK activation is more complex than a simple linear pathway. For example, LIF-triggered ERK1/2 activity in 3T3-L1 adipocytes can occur via both Raf-1-dependent and -independent pro-

[6] P. Rudland and L. Jiménez de Asúa, unpublished results.

cesses (22). In addition, it has been shown that an increase in phosphatidylinositol 3-kinase activity may be involved in ERK activation (45) and that phosphatidylinositol 3-kinase may play a role in prolonging ERK activity (46). Moreover, cytokines such as interferon β or OSM can activate Raf-1 in a Ras-independent manner via increased activity of JAK1 or Tyk2 (47). These findings and our present results support the notion that multiple, temporally distinct pathways can converge on MAPK and that these pathways can be utilized differentially by various stimuli and cell types.

Cyclin D_1 expression is generally regulated by a mitogen-induced Raf/MEK/ERK signaling pathway (26–29). Indeed, the duration of an ERK signal appears to determine whether cells will induce cyclin D_1 expression. Mitogens that only produce a transient ERK activation fail to induce cyclin D_1, whereas growth factors that induce a sustained ERK activation cause continuous maintenance of cyclin D_1 expression (48, 49). Thus, the critical determinant in the induction of cyclin D_1 is the duration, rather than the intensity, of the ERK signal. Our studies show that LIF-stimulated DNA synthesis requires an intact MEK/ERK signaling cascade. However, LIF-stimulated ERK activation is likely not to be linked to the increase in cyclin D_1 expression. In contrast, $PGF_{2\alpha}$-stimulated ERK activation may be directly involved with increasing the expression of cyclin D and ultimately with its mitogenic response. How LIF promotes S phase entry in an ERK-dependent manner and how ERK activation does not result in an increase in cyclin D_1 expression have yet to be elucidated. The results presented here suggest that the different kinetics of MAPK activation may result in a different pattern of G_1 cyclin expression, although alternative explanations based on ERK1/2-independent activation of the cyclin D_1 promoter by $PGF_{2\alpha}$ and not by LIF may be possible.

JAK/STAT signal cascades are known to be involved in responses to cytokines. Our immunofluorescence studies reveal that LIF and OSM trigger a similar pattern of STAT1 cytoplasmic to nuclear translocations after 2 min, attaining a maximum at 10–15 min and declining at 60 min to the basal level, whereas CNTF, IL-6, and $PGF_{2\alpha}$ were without effect. Our experiments to understand the role of LIF induction of DNA synthesis indicated that the effect of LIF is mediated via tyrosine kinase because Na_3VO_4 potentiates LIF's stimulation of DNA synthesis. Furthermore, this result is correlated with the prolonged localization of STAT1 in the nucleus. However, it will be important to elucidate whether STAT1 cytoplasmic-nuclear translocation in conjunction or not with MAPK activation is required for LIF stimulation of DNA synthesis.

LIF is overproduced and secreted by several cancer cells (50, 51) and thus may act as an autocrine stimulator. Moreover, it is known that during oncogenesis different STAT proteins are continuously activated in a variety of cancer cells types (52, 53), and if inhibited the resultant cancer cells grow much more slowly (52, 53, 60, 61). The fact that cytokines stimulate DNA replication through different signaling events may result in carcinomas rapidly eluding the control of growth factor signals, and therefore any therapy targeted to the early signaling events triggered by growth factors may become rapidly ineffective. Thus the elucidation of the molecular mechanism of cytokine action is an important step in dissecting deranged regulatory events leading to malignant transformation and as a second parallel target for mounting a therapeutic blockade aimed at preventing cancer cell proliferation.

Although LIF fails to increase any cyclin D_1 levels, LIF-triggered S phase entry is accompanied by prior increases in cyclin E and cyclin A levels, which are a downstream event(s) to the increase of cyclin D_1 expression (54). Thus, LIF-triggered entry into S phase differs markedly from that triggered by classic mitogens, such as $PGF_{2\alpha}$, bombesin, and other growth factors, which must increase the levels of cyclin Ds to induce S phase entry (36, 55, 56). Whether the low basal levels of cyclin D/CDK4 are sufficient to trigger the next step, phosphorylation of Rb (57), or whether this first stage of Rb phosphorylation is bypassed is unknown at present. Moreover, different findings reveal that cyclin E/CDK2 can induce cellular entry into S phase in the absence of cyclin D/CDK4 activation (56, 58) and that c-Myc and Cdc25A can participate in the activation of the cyclin E-CDK2 complex (59–61). A recent report demonstrates that proliferation of mouse embryonic fibroblasts proceeds relatively normally in the absence of the D-cyclins (62). Kozar *et al.* (62) shows that mouse embryonic fibroblasts from cyclin $D_1^{-/-}D_2^{-/-}D_3^{-/-}$ mice critically depend on CDK2, suggesting that cyclin D-CDK4/6 and cyclin E-CDK2 complexes may perform overlapping functions in "cyclin D-independent" systems. It is established that the initial phosphorylation of the pRb by cyclin D-CDK complexes is required to allow full phosphorylation of the pRb by the cyclin E- and cyclin A-associated kinases (9, 63). However, Kozar *et al.* (62) shows that phosphorylation of pRb on cyclin D-specific sites is not required for further phosphorylation and that cyclin E- and cyclin A-driven phosphorylation is sufficient to allow the expression of E2Fs target genes during cell cycle re-entry. Our study shows that LIF promotes expression of cyclin E and A but not cyclin Ds, as shown in Fig. 6. Furthermore, full phosphorylation of pRb does not take place as cells re-enter the cell cycle. Therefore, our future goal will be to elucidate the role of cyclin E/CDK2 in regulating LIF induction of DNA replication in a cyclin D-independent manner.

In summary, our present work demonstrates that LIF and $PGF_{2\alpha}$ trigger different signaling and molecular events prior to cellular entry into S phase in the same cell system. The importance of this work establishes that stem cell factors like LIF can bypass the normal growth factor-induced Raf/MEK/ERK signaling pathway to cyclin D and activation of CDK4/6, the key event that normally allows progress through the Restriction Point R and commitment to enter the cell cycle (54). LIF and, by implication, stem cell growth factors in general then trigger cellular entry into S phase by partial phosphorylation of Rb through increases in cyclin E and activation of CDK2. Thus the enhancing effect of $PGF_{2\alpha}$ on the induction of cellular entry into S phase mediated by LIF is probably because of the ability of the former mitogen to phosphorylate Rb completely and thereby further reduce its inhibitory activity for the E2F transcription factor required for G_1 to S phase transition (54). Presumably, this synergy is mediated by interactions between those signals generated from the $PGF_{2\alpha}$ receptor that are different from those of LIF, in particular the very early rise in ERK activation and the activation of STAT1. By understanding the molecular mechanisms by which LIF in particular and cytokines in general control normal cell cycle constitutes the basis to unravel the critical cytokine-related signaling events underlying unrestricted cancerous cell division, as well as providing possible therapeutic targets to blockade this second signal transduction pathway in the event that it is necessary to inhibit both growth factor and cytokine signaling pathways to prevent cancer cell growth.

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