Interferon-γ-Mediated Activation of STAT1α Regulates Growth Factor–Induced Mitogenesis

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Abstract

Immunomodulatory cytokines and growth factors act in a complex network to regulate diverse biologic processes. Pretreatment of two types of human vascular pericytes, liver fat–storing cells or glomerular mesangial cells, with IFN-γ dramatically enhanced DNA synthesis in response to PDGF or EGF. IFN-γ by itself had very little effect on DNA synthesis. At least 24-h exposure of the cells to IFN-γ is required for enhancement of growth factor–induced mitogenesis. IFN-γ pretreatment did not influence PDGF or EGF receptor autophosphorylation, activation of phospholipase Cγ1, and phosphatidylinositol 3-kinase, or mitogen-activated protein kinase activity. However, IFN-γ pretreatment markedly potentiated the DNA binding activity of STAT1α in response to PDGF or EGF. Incubation of cells with antisense oligonucleotides targeting STAT1α mRNA resulted in inhibition of DNA synthesis induced by the combination of IFN-γ and PDGF or EGF. These data indicate that interaction between IFN-γ and growth factors at the level of STAT1α results in increased DNA synthesis, and establish a role for STAT1α in this important biologic function of growth factors. (*J. Clin. Invest.* 1996. 98:1218–1230.) Key words: growth factors • DNA synthesis • transcription factors • antisense oligonucleotides

Introduction

Cytokines and growth factors act on target cells in a complex network to elicit their biologic activity. After binding to their cognate cell surface receptors, they transduce signals to the nucleus to stimulate transcription of genes essential for their function in vivo. DNA synthesis and cell proliferation are important biologic functions that are relevant to diverse disease processes, such as carcinogenesis, atherosclerosis, hepatic, renal, and pulmonary fibrosis. Several growth factors, including PDGF and EGF stimulate the proliferation of mesenchymal cells like fibroblasts, liver fat storing cells (FSC)[1] and kidney mesangial cells. The precise mechanism by which cell surface signals are transmitted to the nucleus and ultimately result in cell division, is only partially understood. PDGF and EGF stimulate signaling molecules such as phosphatidylinositol 3 kinase (PI 3-K) and phospholipase Cγ1, (PLCγ1) both of which have recently been shown to be involved in DNA synthesis (1). Recently, another signaling pathway for growth factor receptor tyrosine kinases involving activation of Ras (2–6) has been identified. Stimulation of Ras triggers sequential activation of Raf kinase and MEK leading to activation of mitogen-activated protein kinase (MAPK), which translocate to the nucleus to phosphorylate nuclear proteins to initiate gene transcription (7, 8). PI 3-K itself has been shown to be a target of Ras (9).

Another pathway leading to gene transcription in response to PDGF or EGF independent of Ras/MAPK activation, has been described (10). This pathway comprises a new family of latent transcription factors that are activated in the cytosol by tyrosine phosphorylation, and are translocated to the nucleus where they bind to specific DNA sequences. Tyrosine phosphorylation and dimerization are necessary for DNA binding of this family of transcription factors (11). These molecules have been referred to recently as signal transducers and activators of transcription, or STATs (12). STAT1α, previously referred to as STAT91, is a member of this new family of transcription factors whose activation mediates transcriptional effects of IFN-γ (13, 14). However, STAT1α activation can occur also in response to other cytokines, such as EGF, PDGF, or IL-10 (15–18). The presence of DNA elements in different growth factors and cytokine-inducible genes recognized by STATs indicate that this family of proteins is involved in transcriptional activation. However, the specific biologic effect(s) that result from STAT1α activation in vivo are not known.

The immunomodulatory cytokine IFN-γ is not a classic mitogen for mesenchymal cells, and it has been reported to be antiproliferative for certain cell lines. However, it is frequently expressed in vivo in the presence of other mitogenic cytokines. For example, during an inflammatory response, activated T lymphocytes or monocytes and platelets secrete cytokines including IFN-γ, PDGF, or EGF. In this study, we investigated the effects of IFN-γ on mitogenesis induced by PDGF and EGF in two human primary mesenchymal cell lines, liver FSC

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1. *Abbreviations used in this paper:* FSC, fat-storing cell; GAS, IFN-γ–activated site; GMSA, gel mobility shift analysis; MAPK, mitogen-activated protein kinase; PI 3-K, phosphatidylinositol 3-kinase; PLCγ1, phospholipase Cγ1; RIPA, radioimmunoprecipitation assay; SIE, sis-inducible element; SIF, sis-inducible factor.

and kidney glomerular mesangial cells. We found that IFN-γ dramatically enhances the mitogenic activity of PDGF and EGF, and that this enhanced mitogenicity was not due to increased activation of PI 3-K, PLCγ1, or MAPK pathway. Rather this effect results from the activation of STAT1α. Thus, our studies identify a role for STAT1α activation by IFN-γ in the mitogenic response to PDGF or EGF. These findings have implications relevant to the pathogenesis of inflammatory forms of hepatic and renal injury, as well as other disorders characterized by cell proliferation.

Methods

Cell culture. Human liver FSC were isolated from liver tissue unsuitable for transplantation by collagenase/pronase digestion and centrifugation on stractan gradients, as previously described (19, 20). Cells were cultured in Waymouth's medium supplemented with 17% fetal bovine serum. Normal human kidney tissue unsuitable for transplantation or normal portions from surgical nephrectomy samples were used to culture human glomerular mesangial cells from outgrowths of collagenase-treated glomeruli. Mesangial cells have been extensively characterized by electron microscopy and immunohistochemical staining as described (21). The cells represent a homogeneous population of smooth muscle-like cells free of endothelial or epithelial cell contamination. The cells were grown in Waymouth's medium in the presence of 17% FCS.

Measurement of DNA synthesis. Confluent cells were washed with phosphate-buffered saline and incubated in serum-free medium for 48 h in the absence or presence of IFN-γ, IL-1, or TNF. Growth factors such as PDGF and EGF or thrombin were then added for additional 24 h before pulsing with [^{3}H]thymidine for 4 h. Measurement of thymidine incorporation was performed as described elsewhere (22).

Western blotting. Confluent cells were treated as indicated above, quickly placed on ice and, washed with ice-cold PBS. The monolayer was lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM PMSF, 0.05% [wt/vol] 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 (Bio-Rad Laboratories, Hercules, CA). Identical amounts of protein were separated by SDS-PAGE according to Laemmli (23), and transblotted on a polyvinylidene-difluoride membrane using a semi-dry apparatus (Bio-Rad Laboratories). The membranes were blocked overnight at 4°C with 5% nonfat dry milk in 0.1% PBS-Tween, and sequentially incubated at room temperature with primary and then horseradish peroxidase-conjugated (Gibco-BRL, Grand Island, NY) secondary antibody. Detection was carried out using chemiluminescence according to the manufacturer's protocol (Amersham Corp., Arlington Heights, IL). For immunoprecipitation studies, 300 μg of protein were immunopre-

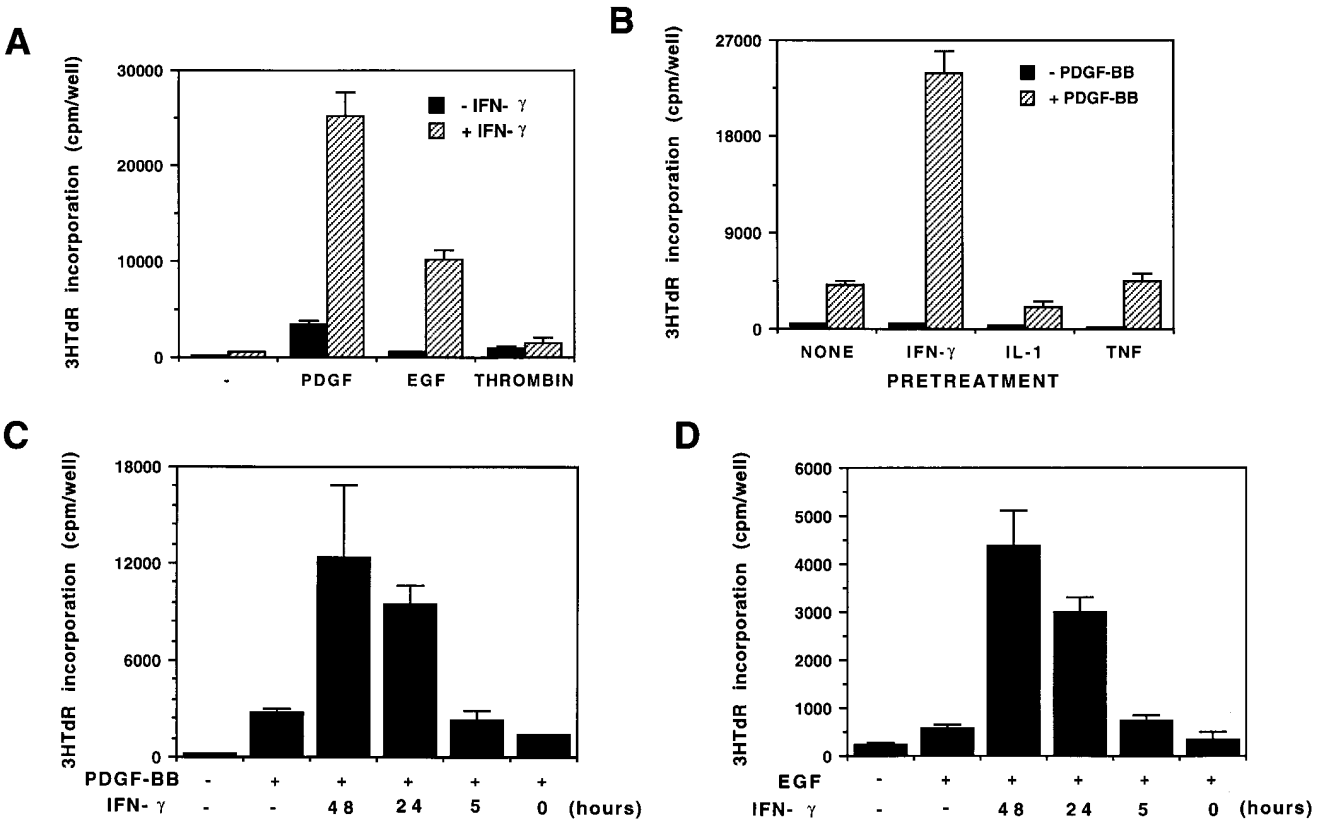


Figure 1. Effect of IFN-γ on growth factor-induced DNA synthesis. (*A*) Confluent FSC were incubated in serum-free medium in the presence (*cross-hatched columns*) or absence (*black columns*) of 1,000 U/ml IFN-γ for 48 h, and then with PDGF-BB (10 ng/ml), EGF (50 ng/ml), or thrombin (5 U/ml) for additional 24 h. Cells were then pulsed with 1 μCi/ml of [^{3}H]thymidine and DNA synthesis was measured as described in Methods. (*B*) Confluent FSC were incubated in serum-free medium in the presence of IFN-γ (1,000 U/ml), IL-1α (15 U/ml), or TNFα (100 U/ml) for 48 h, and then in the presence (*cross-hatched columns*) or absence (*black columns*) of PDGF-BB for additional 24 h, before pulsing and measuring DNA synthesis. (*C*) Confluent FSC were incubated in serum-free medium with IFN-γ (1,000 U/ml) for different periods of times before addition of PDGF-BB (10 ng/ml) for additional 24 h. DNA synthesis was then measured. (*D*) The experiment was performed exactly as in *C*, but the cells were incubated with EGF (100 ng/ml) after IFN-γ pretreatment.

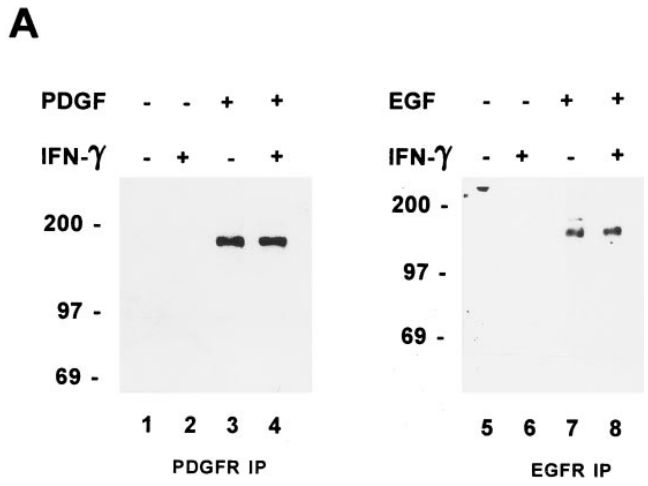


Figure 2. Effect of IFN-γ on PDGF or EGF receptor autophosphorylation and PDGF receptor expression. (*A*) Confluent FSC were incubated in serum-free medium with or without IFN-γ (1,000 U/ml) for 48 h, and then with PDGF-BB (10 ng/ml, lanes *3* and *4*) or EGF (50 ng/ml, lanes *7* and *8*) for 10 min. Cellular proteins were immunoprecipitated using monoclonal antibodies against the PDGF-β-receptor (lanes *1–4*) or the EGF receptor (lanes *5–8*). After washing, the immunobeads were separated by SDS-PAGE and analyzed by immunoblotting using monoclonal antiphosphotyrosine antibodies. Migration of the molecular size marker is indicated in kilodaltons. (*B*) Confluent FSC were incubated in serum-free medium with IFN-γ (1,000 U/ml) for different periods of times. Cellular proteins were separated by SDS-PAGE and analyzed by immunoblotting using a polyclonal antibody against the PDGF β-receptor. Migration of the molecular size marker is indicated in kilodaltons.

cipitated with monoclonal antibodies against the PDGF-β receptor (Genzyme Corp., Cambridge, MA) or against the EGF receptor (Oncogene Science, Manhasset, NY). The immunobeads were washed and then used for Western blot analysis as described above.

Measurement of inositol trisphosphates. Inositol trisphosphates were measured as described in detail elsewhere (24). Confluent FSC were incubated in serum-free medium containing 3 μCi/ml of 2[^{3}H]myo-inositol (Amersham Corp.) in the presence or absence of IFN-γ (1,000 U/ml) for 48 h. 15 mM LiCl was added to the cells for 15 min. PDGF was then added for 5 min. The cells were harvested in 5% trichloroacetic acid and ether extracted. The samples were loaded onto 2-ml AG1X8 anion exchange columns (Bio-Rad Laboratories). The columns were washed with 10 ml of water and 10 ml of 5 mM sodium tetraborate, 60 mM sodium formate. After sequential elution of inositol monophosphate and inositol bisphosphate, inositol trisphosphates were eluted with 10 ml of 0.1 M formic acid, 0.8 M ammonium formate. 5 ml of this fraction were mixed with scintillation fluid and counted in a beta counter.

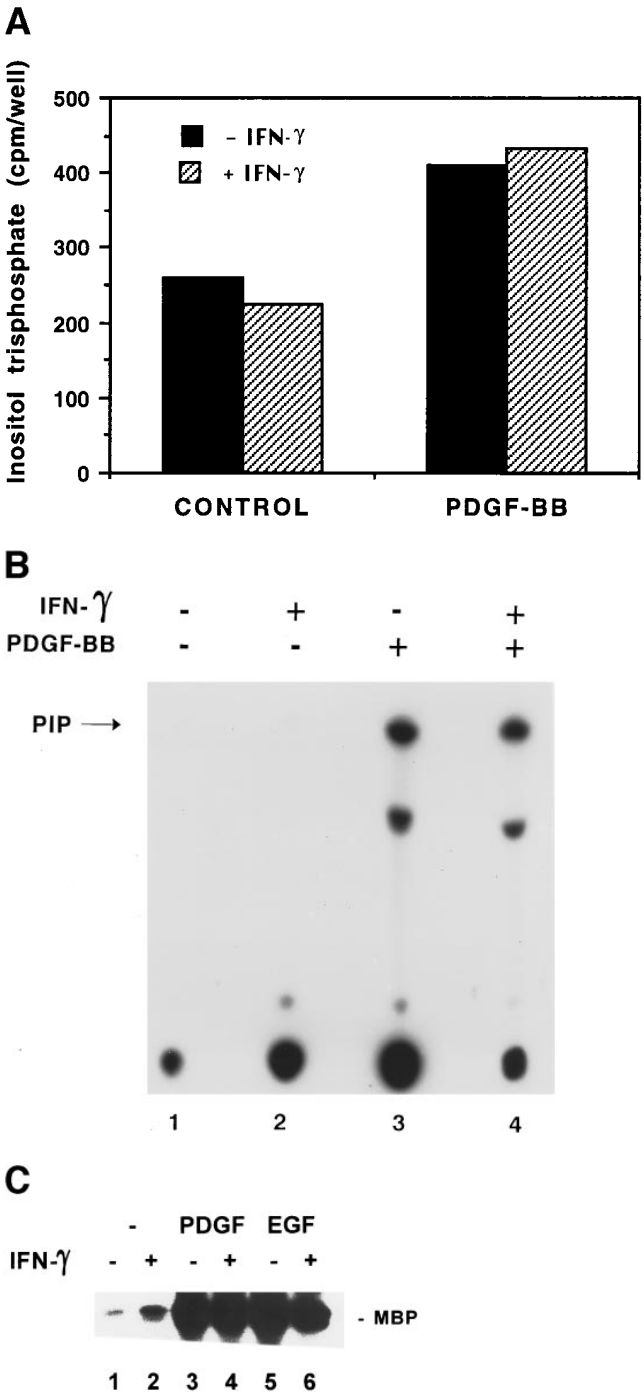


Figure 3. Effect of IFN-γ on growth factor stimulated early signaling. (*A*) Confluent FSC were incubated in serum-free medium with or without IFN-γ (1,000 U/ml) for 48 h, and then with PDGF-BB (10 ng/ml) for 10 min. Cells were lysed and inositol phosphates were separated by anion exchange chromatography as described in Methods. (*B*) FSC were treated as described in *A*. Cellular proteins were immunoprecipitated with antiphosphotyrosine antibodies, and PI 3-K activity was measured on the immunobeads as described in Methods. The arrow indicates 3-OH phosphorylated phosphatidylinositol. (*C*) Confluent FSC were incubated in serum-free medium in the absence (lanes *1*, *3*, and *5*) or presence (lanes *2*, *4*, and *6*) of IFN-γ (1,000 U/ml) for 48 h, then PDGF-BB (10 ng/ml, lanes *3* and *4*) or EGF (100 ng/ml, lanes *5* and *6*) were added for 10 min. Cell lysates were immunoprecipitated with polyclonal anti–MAPK antibodies, and the immunobeads were assayed for myelin basic protein kinase activity as described in Methods. The figure shows the band corresponding to myelin basic protein after separation on 15% SDS-PAGE and autoradiography of the gel.

Phosphatidylinositol 3-kinase activity. Cells were incubated in serum-free medium in the presence or absence of IFN-γ (1,000 U/ml) for 48 h, and then stimulated with PDGF-BB (10 ng/ml) for 10 min. PI 3-K assay was performed as described elsewhere (25, 26). Briefly, the cells were lysed in RIPA buffer and equivalent amounts of protein were immunoprecipitated using an agarose-conjugated antiphosphotyrosine antibody (Oncogene Science). After washing, the immunobeads were resuspended in 50 μl of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 mM EGTA. 0.5 μl of 20 mg/ml phosphatidylinositol (Sigma Chemical Co., St. Louis, MO) was added, mixed, and incubated at 25°C for 10 min. 1 μl of 1 M $MgCl_2$ and 10 μCi of [γ-^{32}P]ATP were then added simultaneously, and incubated at 25°C for additional 10 min. The reaction was stopped by addition of 150 μl of chloroform/methanol/37% HCl 10:20:0.2. The samples were extracted with chloroform and dried. Radioactive lipids were separated by thin-layer chromatography and developed in 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 was confirmed using high-pressure liquid chromatography (25).

Mitogen-activated protein kinase assay. MAPK activity was measured by an in vitro kinase assay after immunoprecipitation, as described elsewhere (27). RIPA lysates from HSC (100 μg of protein) were immunoprecipitated with polyclonal antibodies recognizing both p44 and p42 MAPK isoforms (a kind gift of Dr. M.J. Dunn, Medical College of Wisconsin, Milwaukee, WI) and protein A sepharose. After washing, the immunobeads were incubated in a buffer containing 10 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 1 mM dithiotreitol, 1 mM Na_3VO_4, 1 μCi [γ-^{32}P]ATP, and 0.5 mg/ml myelin basic protein for 30 min at 30°C. At the end of the incubation, 15 μl of the reaction mixture were spotted onto phosphocellulose disks, washed four times in 1% phosphoric acid, and the radioactivity counted in a beta-counter. Another aliquot (10 μl) of the reaction mixture was run on 15% SDS-PAGE (23). After electrophoresis, the gel was dried and autoradiographed.

Preparation of nuclear extracts. Nuclear extracts were prepared as described by Andrews and Faller (28). FSC in 150-mm dishes were washed twice, then scraped in PBS using a rubber policeman. The cell pellet was resuspended in a buffer containing 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 2 mM Na_3VO_4, 0.5 mM PMSF, 0.05% aprotinin, and 0.1% Triton X-100, and gently homogenized using a glass Pasteur pipette. The cell lysate was spun at 4°C at 1,000 *g*, and the pellet resuspended in the same buffer and spun again. The pellet was then resuspended in 20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.05% aprotinin, and 2 mM Na_3VO_4, and mixed on a rocking platform for 30 min at 4°C. The mixture was then spun at 10,000 *g* and the supernatant saved. Integrity and purity of the nuclei was assessed under light microscopy. Protein concentration of the nuclear extract was then determined and aliquots were stored at −80°C.

Gel mobility shift assay. Double-stranded oligonucleotides corresponding to the high affinity m67 (5′-CAGTTCCCGTCAATC-3′)

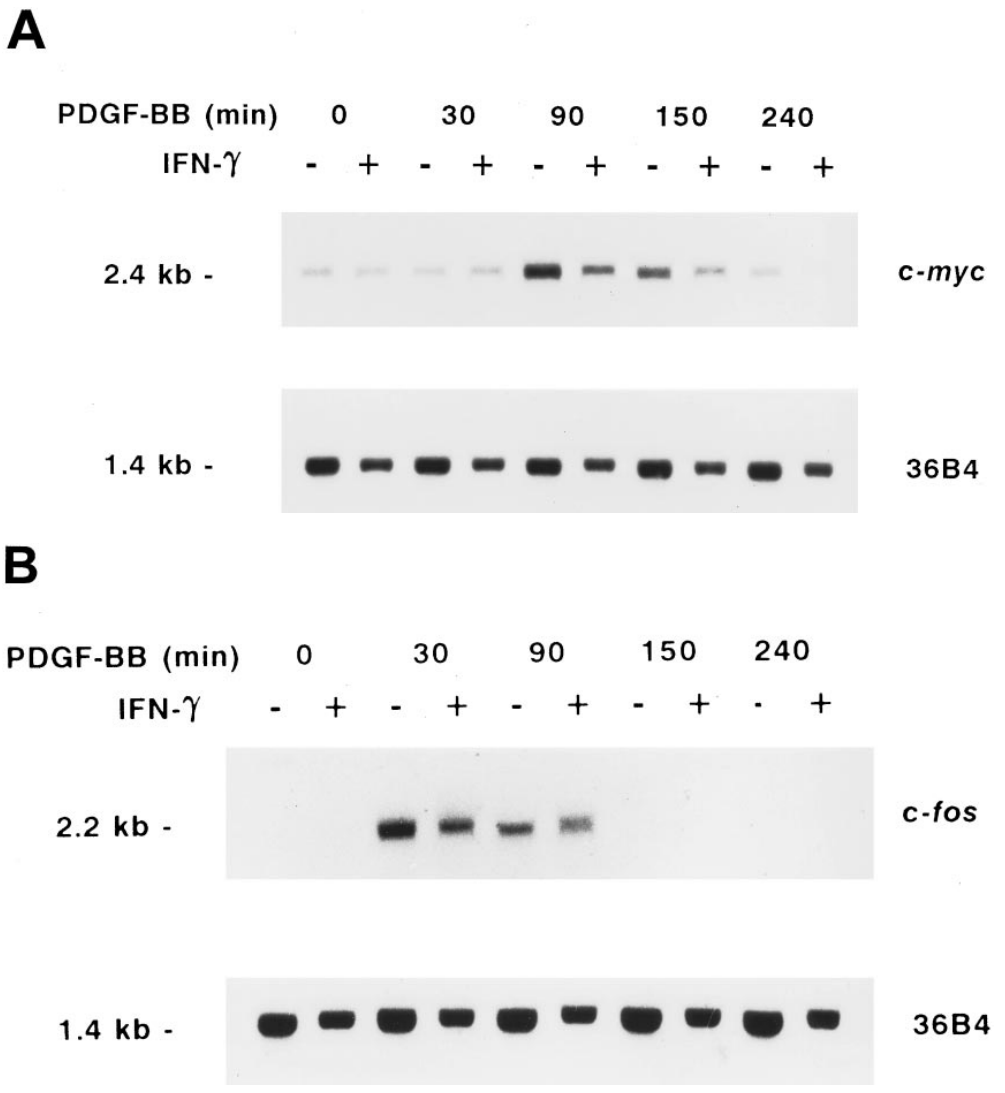


Figure 4. Effect of IFN-γ on PDGF-stimulated c-*myc* and c-*fos* expression. (*A*) Confluent FSC were incubated in serum-free medium with or without IFN-γ (1,000 U/ml) for 48 h, and then with PDGF-BB (10 ng/ml) for the indicated periods of time. 15 μg of total RNA were separated on an agarose-formaldehyde gel, blotted on a membrane and hybridized with a radiolabeled cDNA encoding for c-*myc* (*A*) or c-*fos* (*B*). The probe was then removed and the blots were rehybridized with a cDNA encoding for the ribosomal protein 36B4 (control probe).

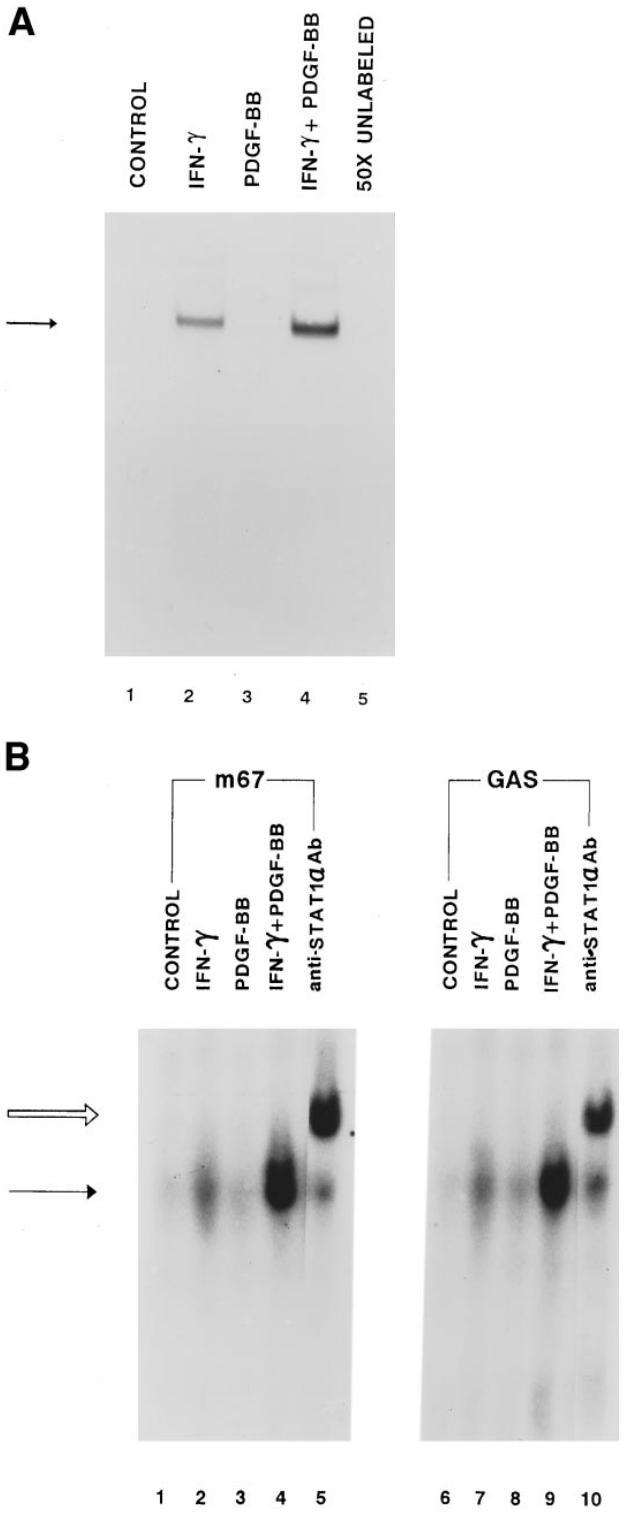


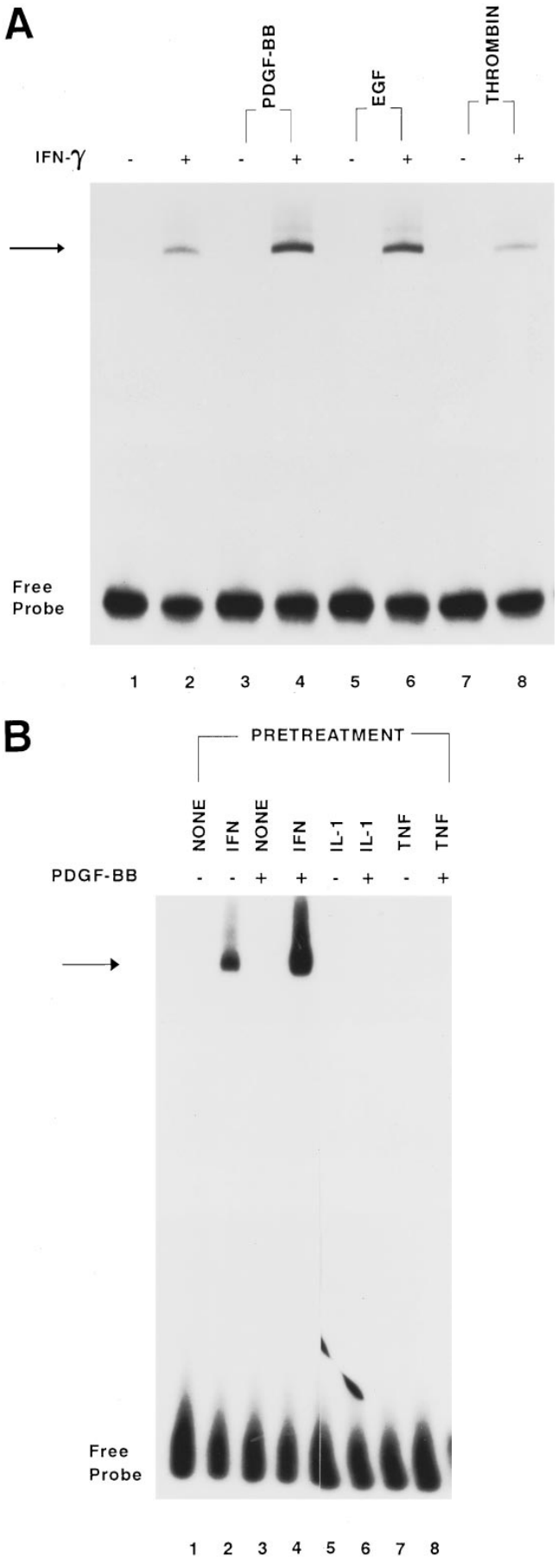
Figure 5. Stimulation of PDGF-induced DNA binding activity of STAT1α after IFN-γ treatment. (*A*) FSC were incubated with IFN-γ (1,000 U/ml, lanes *2* and *4*) for 48 h, and then with PDGF-BB (10 ng/ml, lanes *3* and *4*) for 15 min. Nuclear extracts were prepared, and 15 μg of nuclear protein were used in a GMSA with a labeled m67 probe as described in Methods. In lane *5*, the same sample as in lane *4* was incubated in the presence of a 50-fold excess of unlabeled m67 oligonucleotide. The arrow indicates the protein-DNA complex. Prolonged exposure showed that PDGF alone (lane *3*) increases DNA binding activity over control (lane *1*) (*B*). The cells were incubated with IFN-γ and PDGF-BB as described in Fig. 4 *A*. Total cell extracts were used in GMSA using either m67 (lanes *1–5*) or GAS (lanes *6–10*) radiolabeled probes. In lanes *5* and *10*, samples shown in lanes *4* and *9*, respectively, were preincubated with a monoclonal antibody directed against STAT1α. The black arrow indicates the protein-DNA complex. The open arrow indicates the further retardation of the complex ("supershift") in the presence of antibodies.

(29) and to the Ly-6E IFN-γ–activated site (GAS) (5′-ATATTCCT-GTAAGTG-3′) (30) were synthesized using a 329 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). The single-stranded oligonucleotides were annealed and labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP. Labeled DNA was separated from the unincorporated radioactivity using an ion-exchange column (Schleier and Schuell, Keene, NH). 8–15 μg of nuclear extracts or, where specified, total cell extracts were incubated in binding buffer (final concentrations 35 mM Hepes, pH 7.8, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol) containing 10 μg/ml poly dIdC (Pharmacia Fine Chemicals, Piscataway, NJ) and 50,000–100,000 cpm of radiolabeled DNA for 30 min at 25°C. The samples were then separated on a native 5% polyacrylamide gel (50 mM Tris, 380 mM glycine, 10% glycerol). After electrophoresis, the gel was dried and autoradiographed. For "supershift" experiments, the cell extracts were incubated with a monoclonal anti-STAT1α/STAT1β antibody (Transduction Laboratories, Lexington, KY) for 30 min on ice before the binding reaction.

Antisense oligonucleotides. Antisense phosphorothioate-modified oligonucleotides encompassing the translation initiation site of the STAT1α mRNA (31) were used (sequence: 5′-TACCACT-GAGACATCCTGCC-3′). The sense sequence was used as control (5′-GGCAGGATGTCTCAGTGGTA-3′). For experiments, the cells were washed with PBS and incubated overnight with a mixture of oligonucleotides (final concentration 1 μM) and cationic lipids (Lipofectamine®; Gibco BRL, final concentration 10 μg/ml) in serum-free medium. The liposome/DNA mixture was then removed and cells were incubated with IFN-γ for 24 h followed by PDGF or EGF for additional 24 h before pulsing with thymidine. For Western blot analysis of STAT1α expression, the cells were incubated with IFN-γ for 24 h and RIPA lysates were used as described above.

Results

Interferon-γ potentiates growth factor-induced mitogenesis. Interferon-γ is a poor mitogen for FSC and has been reported to inhibit DNA synthesis in rat FSC (32). We tested the effect of exposure of human liver FSC to IFN-γ on the mitogenic response to PDGF or EGF, known mitogens for these and other mesenchymal cells (22). FSC were pretreated with IFN-γ for 48 h followed by incubation with PDGF or EGF for 24 h and pulsing with [^{3}H]thymidine. EGF or PDGF stimulated DNA synthesis approximately two- and ten-fold, as compared to cells treated with control vehicle (Fig. 1 *A*). Preincubation with IFN-γ resulted in a dramatic potentiation of the effects of PDGF or EGF (Fig. 1 *A*). The increased DNA synthesis in cells pretreated with IFN-γ before addition of the growth factor increased 7- to 20-fold in comparison to cells treated with PDGF or EGF alone (Fig. 1 *A*). Cells treated with IFN-γ alone for the duration of the experiment, i.e., 72 h before pulsing with thymidine showed negligible increase in DNA synthesis (twofold over basal, Fig. 1 *A*). Preincubation with IFN-γ did not potentiate thrombin-induced DNA synthesis (Fig. 1 *A*) suggesting that the potentiating effect of IFN-γ is selective for mitogens with receptor tyrosine kinases. Immunoinflammatory cytokines other than IFN-γ also exert biologic effects on FSC (20). We tested the effects of IL-1α or TNFα on growth



factor–induced DNA synthesis. As shown in Fig. 1 *B*, preincubation of cells with either of these cytokines did not potentiate PDGF-induced DNA synthesis. At least 24 h preincubation with IFN-γ is required for the potentiating effects of PDGF or EGF (Fig. 1, *C* and *D*). A 5-h preincubation or the simultaneous addition of IFN-γ and either PDGF or EGF failed to elicit potentiation of DNA synthesis (Fig. 1, *C* and *D*).

Potentiation of DNA synthesis by IFN-γ occurs without changes in PDGF β-receptor early signaling or MAP kinase activity. We studied the early signaling pathways that follow the binding of PDGF or EGF to their cognate receptors to investigate the mechanisms underlying the potentiation of the mitogenic effect. The first event that follows agonist-receptor interaction is receptor dimerization and autophosphorylation on tyrosine residues. Tyrosine phosphorylation of the PDGF β-receptor and of the EGF-receptor was unaffected when cells were pretreated with IFN-γ for 48 h before addition of the corresponding ligands, as assessed by immunoprecipitation of the receptors followed by antiphosphotyrosine immunoblotting (Fig. 2 *A*). In addition, the level of the PDGF β-receptor evaluated by Western blotting, was not modified by pretreatment of the cells with IFN-γ for different periods of time before the addition of PDGF (Fig. 2 *B*). These results indicate that prolonged exposure of cells to IFN-γ did not modify the phosphorylation state or the expression of growth factor receptors.

Upon activation, the autophosphorylated PDGF β-receptor physically associates with several signaling molecules (33). Recent studies have pointed out the importance of some of these molecules in mediating the mitogenic effects of PDGF. Activation of phospholipase Cγ1 results in hydrolysis of phosphatidylinositol bisphosphate and generation of inositol trisphosphate and diacylglycerol, which stimulate an increase in cytosolic calcium concentration and activation of protein kinase C, respectively (34). We measured the levels of inositol trisphosphate as an index of PLCγ1 activation in FSC pretreated with IFN-γ before the addition of PDGF (Fig. 3 *A*). IFN-γ pretreatment did not potentiate the increase in inositol trisphosphate production induced by PDGF. Another pathway that is involved in PDGF-induced mitogenesis is activation of PI 3-K (35). We measured PI 3-K activity in PDGF-stimulated cells after immunoprecipitation with antiphosphotyrosine antibodies (Fig. 3 *B*). Pretreatment with IFN-γ did not potentiate PI3-K activity in response to PDGF.

Most signals generated by ligand–receptor interaction at the cell surface result in stimulation of mitogenesis through the activation of MAPK (8). Recent studies indicate that cell growth may be partially modulated at the level of MAPK. For example, agents which elevate cyclic AMP levels may inhibit

Figure 6. Effects of different combination of cytokines and growth factor on DNA binding activity to m67. (*A*) FSC were incubated with IFN-γ (1,000 U/ml, lanes *2*, *4*, *6*, and *8*) for 48 h, and then with PDGF-BB (10 ng/ml, lanes *3* and *4*), EGF (50 ng/ml, lanes *5* and *6*), or thrombin (5 U/ml, lanes *7* and *8*) for 15 min. Total cell extracts were used in GMSA using m67 probe. The arrow indicates the protein–DNA complex. The unbound probe is also shown. (*B*) FSC were pretreated for 48 h with IFN-γ (1,000 U/ml, lanes *2* and *4*), IL-1α (15 U/ml, lanes *5* and *6*) or TNFα (100 U/ml, lanes *7* and *8*) and then with PDGF-BB (10 ng/ml, lanes *2*, *4*, *6*, and *8*) for 15 min. Total cell extracts were used in GMSA using m67 probe. The arrow indicates the protein–DNA complex. The unbound probe is also shown.

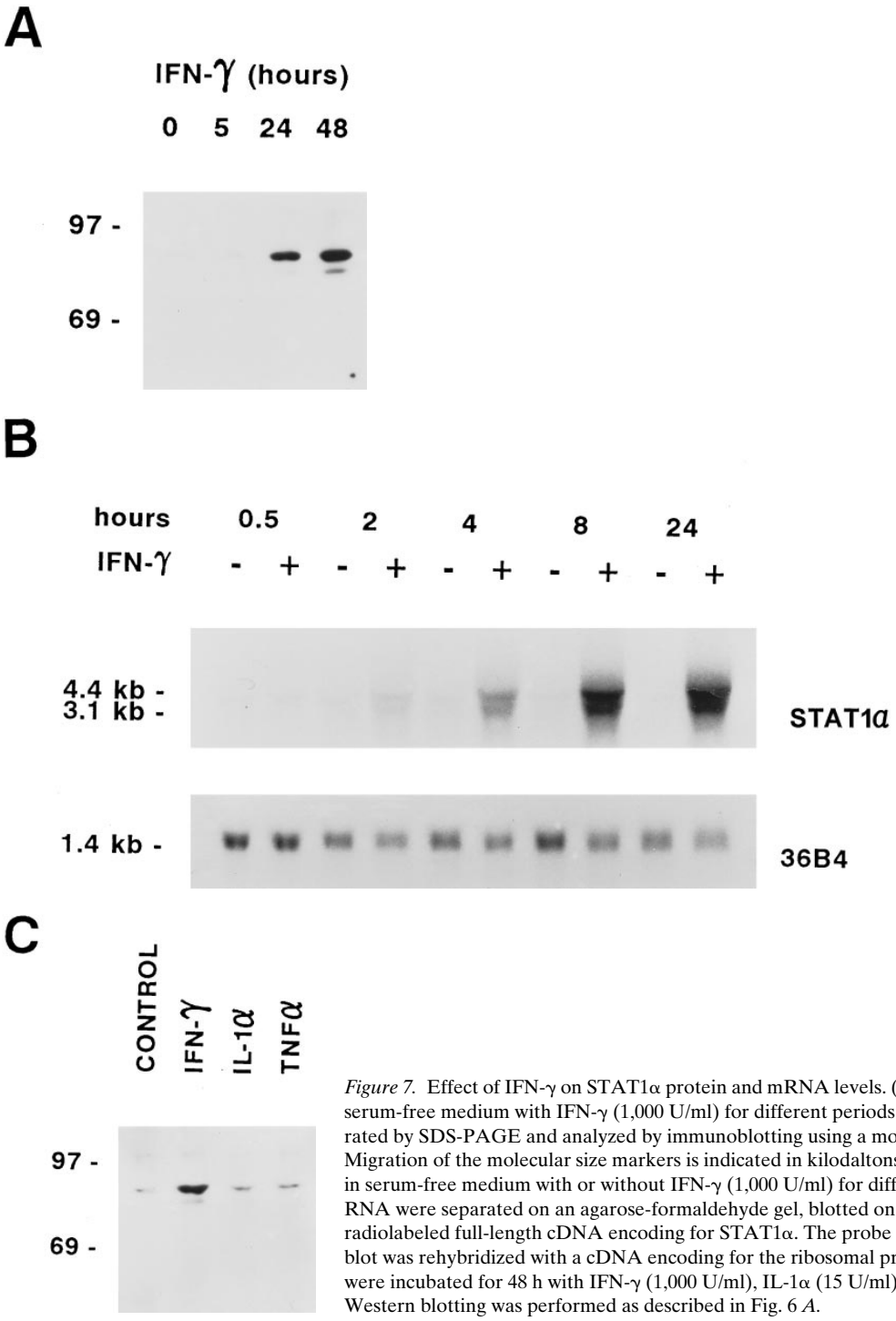


Figure 7. Effect of IFN-γ on STAT1α protein and mRNA levels. (*A*) Confluent FSC were incubated in serum-free medium with IFN-γ (1,000 U/ml) for different periods of time. Cellular proteins were separated by SDS-PAGE and analyzed by immunoblotting using a monoclonal antibody against STAT1α. Migration of the molecular size markers is indicated in kilodaltons. (*B*) Confluent FSC were incubated in serum-free medium with or without IFN-γ (1,000 U/ml) for different periods of time. 15 μg of total RNA were separated on an agarose-formaldehyde gel, blotted on a membrane, and hybridized with a radiolabeled full-length cDNA encoding for STAT1α. The probe was then removed and the stripped blot was rehybridized with a cDNA encoding for the ribosomal protein 36B4 (control probe). (*C*) FSC were incubated for 48 h with IFN-γ (1,000 U/ml), IL-1α (15 U/ml), or TNFα (100 U/ml). STAT1α Western blotting was performed as described in Fig. 6 *A*.

cell growth via inhibition of Raf kinase which is an upstream regulator of MAPK (36, 37). We tested the effect of IFN-γ pretreatment on growth factor stimulated MAPK activity. Addition of PDGF or EGF to FSC resulted in an approximately fourfold increase in MAPK activity (Fig. 3 *C*), which was not upregulated by pretreatment of the cells with IFN-γ. Other important growth factor-induced early signal transduction events include expression of c-*myc* and c-*fos* protooncogenes (38, 39). PDGF stimulated c-*myc* and c-*fos* expression in FSC (Fig. 4). However, IFN-γ pretreatment did not modify the increased protooncogene expression in response to PDGF. Taken together, these data indicate that IFN-γ pretreatment did not influence the effect of growth factors on early signaling.

Interferon-γ pretreatment potentiates activation of STAT1α in response to PDGF. STAT1α was initially characterized as the gamma-interferon activated factor (GAF), that binds to the GAS (13, 14). It has recently been reported that tyrosine phosphorylated, and therefore activated STAT1α is expressed after stimulation of cells with PDGF or EGF (15–18). In addition, STAT1α is part of the complex of transcription factors

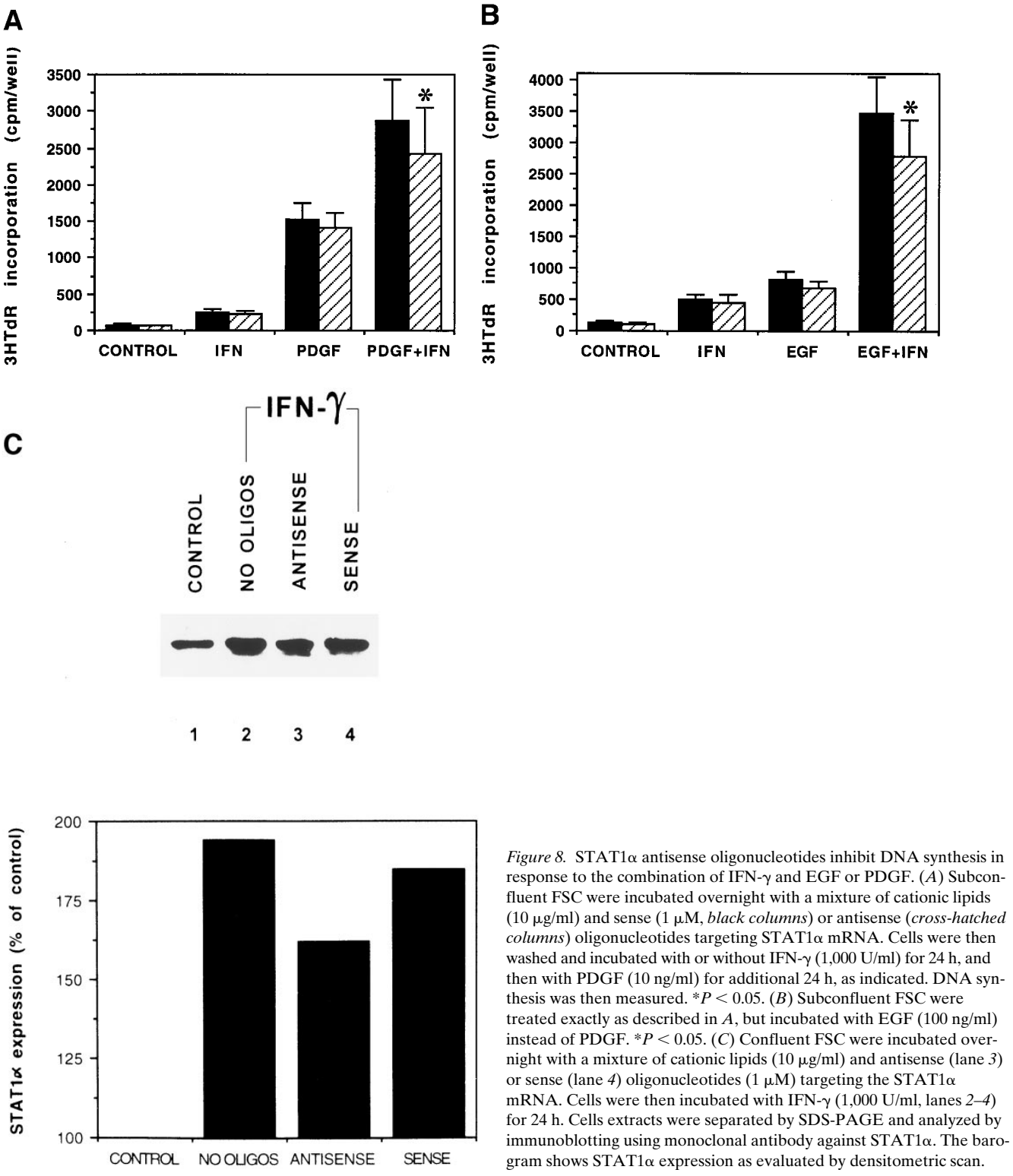


Figure 8. STAT1α antisense oligonucleotides inhibit DNA synthesis in response to the combination of IFN-γ and EGF or PDGF. (*A*) Subconfluent FSC were incubated overnight with a mixture of cationic lipids (10 μg/ml) and sense (1 μM, *black columns*) or antisense (*cross-hatched columns*) oligonucleotides targeting STAT1α mRNA. Cells were then washed and incubated with or without IFN-γ (1,000 U/ml) for 24 h, and then with PDGF (10 ng/ml) for additional 24 h, as indicated. DNA synthesis was then measured. $*P < 0.05$. (*B*) Subconfluent FSC were treated exactly as described in *A*, but incubated with EGF (100 ng/ml) instead of PDGF. $*P < 0.05$. (*C*) Confluent FSC were incubated overnight with a mixture of cationic lipids (10 μg/ml) and antisense (lane *3*) or sense (lane *4*) oligonucleotides (1 μM) targeting the STAT1α mRNA. Cells were then incubated with IFN-γ (1,000 U/ml, lanes *2–4*) for 24 h. Cells extracts were separated by SDS-PAGE and analyzed by immunoblotting using monoclonal antibody against STAT1α. The barogram shows STAT1α expression as evaluated by densitometric scan.

which binds the sis-inducible element (SIE) of the c-*fos* promoter (17, 29). Therefore, STAT1α activation is shared by the signaling pathways of PDGF, EGF, and IFN-γ.

We performed gel mobility shift assay (GMSA) using nuclear extracts from FSC and a high affinity DNA site (m67) derived from the SIE of the human c-*fos* promoter (29). It is known that IFN-γ, as well as other soluble factors, can induce DNA binding activity to the SIE. Also in FSC, this cytokine rapidly induced m67-binding activity with a peak at 15 min, followed by persistant activation, although at reduced level, for as long as 48 h, (data not shown). As shown in Fig. 5 *A*, the DNA-binding activity to labeled m67 in response to PDGF was dramatically potentiated by interferon-γ pretreatment (compare lanes *3* and *4*). Note that pretreatment with IFN-γ alone for 48 h

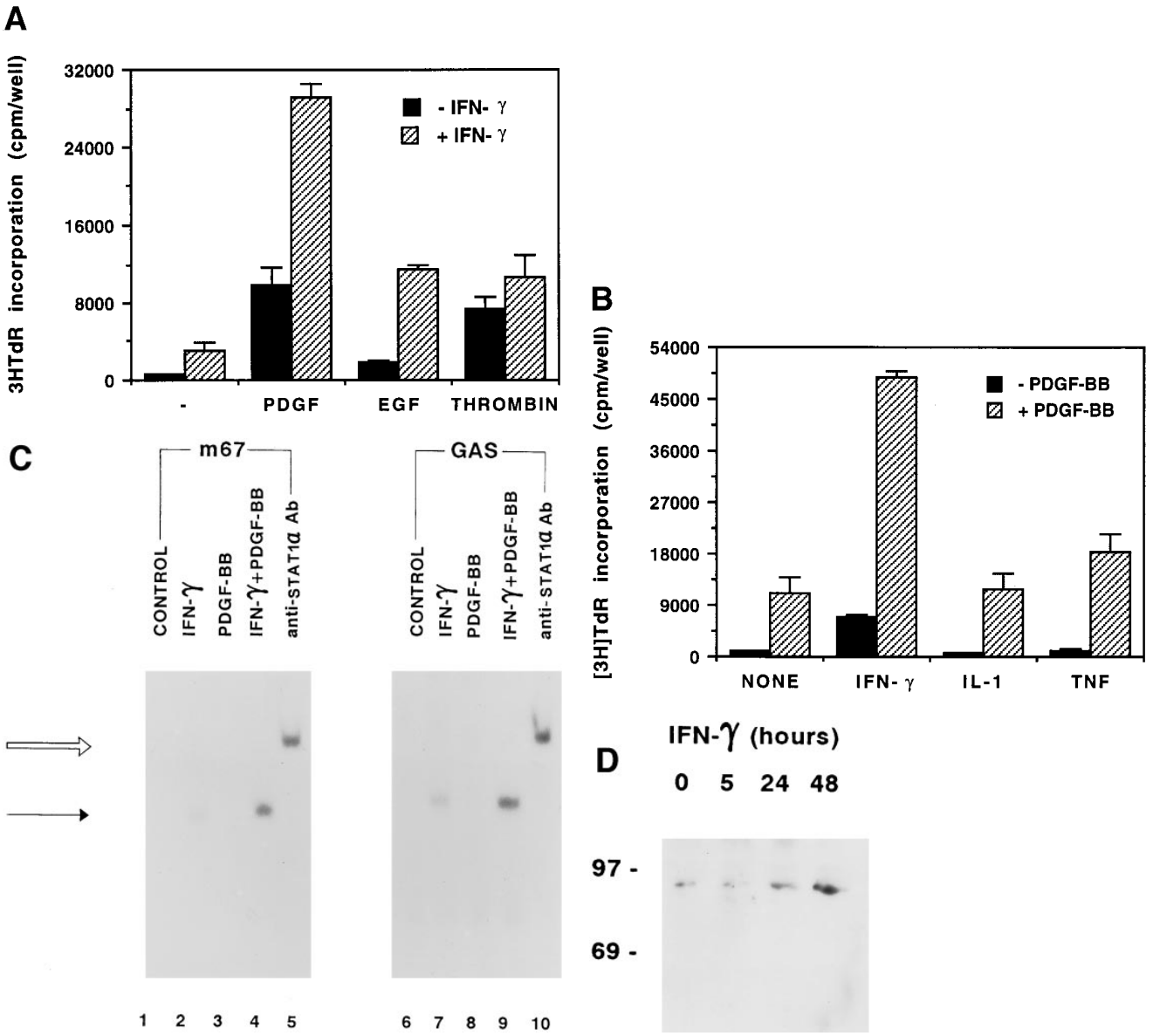


Figure 9. Effects of IFN-γ pretreatment in human glomerular mesangial cells. (*A*) Confluent mesangial cells were incubated in serum-free medium in the presence (*cross-hatched columns*) or absence (*black columns*) of 1,000 U/ml IFN-γ for 48 h, and then with PDGF-BB (10 ng/ml), EGF (50 ng/ml) or thrombin (5 U/ml) for additional 24 h. Cells were then pulsed with 1 μCi/ml of [^{3}H]thymidine and DNA synthesis was measured as described in Methods. (*B*) Confluent mesangial cells were incubated in serum-free medium in the presence of IFN-γ (1,000 U/ml), IL-1α (15 U/ml), or TNFα (100 U/ml) for 48 h, and then in the presence (*cross-hatched columns*) or absence (*black columns*) of PDGF-BB for additional 24 h, before pulsing and measuring DNA synthesis. (*C*) Human mesangial cells were incubated with IFN-γ and PDGF-BB exactly as described in Fig. 4 *A* for FSC. Total cell extracts were used in GMSA using either m67 (lanes *1–5*) or GAS (lanes *6–10*) radiolabeled probes. In lanes *5* and *10*, samples shown in lanes *4* and *9*, respectively, were preincubated with a monoclonal antibody directed against STAT1α. The black arrow indicates the protein-DNA complex. The open arrow indicates the further retardation of the complex ("supershift") in the presence of antibodies. (*D*) Confluent mesangial cells were incubated in serum-free medium with IFN-γ (1,000 U/ml) for different periods of times. Cellular proteins were separated by SDS-PAGE and analyzed by immunoblotting using a monoclonal antibody against STAT1α. Migration of the molecular size markers is indicated in kilodaltons.

(lane *2*) resulted in a higher DNA binding activity than PDGF alone, but the combination of the two factors produced a marked increase in the binding activity (compare lanes *2* and *3* with lane *4*). An increase in DNA binding activity measured by phosphoimage analysis (not shown) was also observed in cells treated with PDGF alone compared to controls (two- to threefold).

EGF treatment of A431 cells results in the formation of three complexes that bind to the m67 oligonucleotide, referred to as SIF-(sis-inducible factor) A, SIF-B, and SIF-C (40). Recent evidence indicates that two proteins participate in the formation of the SIF complexes, namely STAT1α and STAT3 (41). However, only STAT1α is activated by IFN-γ as well as by EGF or PDGF, whereas STAT3 is activated by EGF and other agents, such as IL-6 and lipopolysaccaride (41, 42). To confirm that STAT1α is involved in determining DNA binding activity to m67 in cells pretreated with IFN-γ followed by

PDGF, we performed GMSA using either m67 or GAS element (30), which binds only STAT1α. As shown in Fig. 5 *B*, a synergistic effect between IFN-γ and PDGF in the induction of DNA binding activity was observed using both elements. This finding indicates that activation of STAT1α is at least partially responsible for the DNA binding activity. To further confirm the involvement of STAT1α, extracts from cells treated with IFN-γ for 48 h and then with PDGF were incubated with monoclonal antibodies directed against STAT1α followed by GMSA. Addition of antibodies resulted in a supershift of the great majority of the DNA-protein complex, indicating that the DNA-binding activity is indeed dependent on activated STAT1α (Fig. 5 *B*, compare lane *5* with *4* and lane *10* with *9*).

Selected combinations of cytokines that potentiate DNA synthesis result in further activation of STAT1α. To determine if the potentiation of STAT1α activation occurs in response to different combinations of cytokines, FSC were pretreated with IFN-γ for 48 h followed by PDGF, EGF or thrombin (Fig. 6 *A*). EGF was almost as potent as PDGF in potentiating the DNA binding activity of STAT1α. Thrombin, on the other hand, failed to increase the binding activity of this transcription factor. As described above, IFN-γ pretreatment also did not potentiate the mitogenic effect of thrombin (see Fig. 1 *A*). We then studied the effect of prolonged incubation with other cytokines on PDGF-dependent STAT1α activation. As shown in Fig. 6 *B*, pretreatment of FSC with IL-1α or TNFα did not affect to any extent the DNA binding activity to m67 in response to PDGF. Taken together, these experiments indicate that only pretreatment with IFN-γ potentiates STAT1α activation, and that IFN-γ is the only cytokine which synergizes with growth factors to enhance mitogenesis.

Interferon-γ upregulates STAT1α protein and mRNA levels. At least 24 h pretreatment of FSC with IFN-γ is required for potentiation of the mitogenic effects of PDGF or EGF (see Fig. 1, *C* and *D*). To identify the signaling mechanism involved, we studied the kinetics of STAT1α expression. FSC were incubated with IFN-γ for different periods of time and STAT1α expression was analyzed by Western blotting of total cellular proteins (Fig. 7 *A*). Serum-starved FSC express very low level of STAT1α. IFN-γ treatment caused a time-dependent increase in STAT1α with maximal effects at 48 h. The expression of the short 84-kD spliced variant of STAT1α, referred to as STAT1β (31), was also increased, but to a much lower extent. Incubation with IFN-γ was also associated with increased steady-state levels of STAT1α mRNA (Fig. 7 *B*). Increase in STAT1α mRNA levels was evident after 2 h and continued to increase for at least 24 h. The effects on STAT1α expression were specific for IFN-γ. In fact, STAT1α was not upregulated even after 48 h incubation with IL-1 or TNFα (Fig. 7 *C*).

STAT1α antisense oligonucleotides inhibit DNA synthesis in cells treated with IFN-γ and EGF. To confirm that the activation of STAT1α is involved in the potentiation of the mitogenic effects of growth factors in cells pretreated with IFN-γ, antisense oligonucleotides targeting the translation initiation site of the STAT1α mRNA were used, and a sense sequence was used as a control. A cationic lipid system was used to enhance delivery of oligonucleotides to the cells. [^{3}H]thymidine incorporation was measured in FSC preincubated in the presence of antisense or sense oligonucleotides followed by sequential incubation either with IFN-γ and then PDGF or EGF, or with PDGF or EGF alone. Preincubation with STAT1α antisense oligonucleotides significantly reduced DNA synthesis in response to the combination of IFN-γ and growth factors (Fig. 8, *A* and *B*). The inhibitory effect on DNA synthesis ranged between 15 and 30% and was slightly lower with PDGF than with EGF. Thymidine incorporation in response to either PDGF or EGF alone was not significantly reduced by treatment with antisense oligonucleotides. More importantly, this effect was much lower than that observed when a combination of IFN-γ and growth factors was used, thus indicating that interfering with STAT1α inhibits the potentiation of DNA synthesis caused by IFN-γ pretreatment. Thymidine incorporation in unstimulated cells or in cells treated with IFN-γ alone was not affected by antisense oligonucleotides, thus ruling out a toxic or nonspecific effect.

Since antisense oligonucleotides resulted in only a partial reduction of DNA synthesis stimulation by IFN-γ and growth factors, we determined the effect of similar concentrations of oligonucleotides on the protein levels of STAT1α. FSC were transfected with antisense or sense oligonucleotides, and then incubated with IFN-γ for 24 h. Fig. 8 *C* shows that STAT1α protein levels were reduced by antisense pretreatment, whereas no effect was observed in cells treated with sense oligonucleotides. Densitometric scan of the gel showed ~ 25% reduction of STAT1α levels in the presence of antisense oligonucleotides, a figure comparable to the effects on DNA synthesis. These results demonstrate that the reduction of the levels of STAT1α is associated with significant inhibition of DNA synthesis in cells treated sequentially with IFN-γ and PDGF or EGF.

Potentiation of DNA synthesis by IFN-γ occurs in different mesenchymal cells. Liver FSC represent a relatively specialized mesenchymal cell type. We carried out experiments to determine if the potentiation of mitogenesis by IFN-γ and the activation of STAT1α were shared by different cell types. Human glomerular mesangial cells were treated with IFN-γ for 48 h, followed by PDGF. As shown in Fig. 9 *A*, the mitogenic effect of PDGF was potentiated by IFN-γ pretreatment as observed in liver FSC. The two immunoinflammatory cytokines, IL-1α and TNFα did not potentiate the effect of PDGF on DNA synthesis (Fig. 9 *B*). GMSA carried out using mesangial cell extracts showed potentiation of the binding activity to m67 or GAS by the combination of IFN-γ followed by PDGF, and the great majority of the complex was supershifted by anti-STAT1α antibodies (Fig. 9 *C*). Finally, STAT1α protein expression was upregulated by IFN-γ in a time-dependent fashion as observed in FSC (Fig. 9 *D*).

Pretreatment of another primary mesenchymal cell line, human foreskin fibroblasts, also demonstrated that IFN-γ potentiates the effects of PDGF and EGF on DNA synthesis (data not shown). These results demonstrate that the potentiation of the mitogenic effects of PDGF and EGF by IFN-γ is observed in at least three primary human mesenchymal cell lines, and that activation of STAT1α most likely represents a common mechanism that mediates this effect of IFN-γ.

Discussion

This study establishes a role for STAT1α activation in mediating the dramatic potentiation of DNA synthesis upon exposure of cells to IFN-γ and PDGF or EGF. This conclusion is based on the following experimental observations. (*a*) Pretreatment of FSC or mesangial cells with IFN-γ, but not IL-1

or TNF markedly potentiates DNA synthesis induced by PDGF or EGF. (*b*) IFN-γ pretreatment results in marked increase in DNA binding activity to m67 and GAS elements that can be supershifted in the presence of STAT1α antibody. (*c*) IFN-γ upregulates STAT1α mRNA and protein. (*d*) Antisense but not sense oligonucleotides to STAT1α reduce STAT1α abundance and DNA synthesis. Furthermore, the data show that the priming effect of IFN-γ does not depend on early signals involved in growth factor-induced mitogenesis. In fact, PDGF and EGF receptor activation as measured by their level of tyrosine phosphorylation was not affected by IFN-γ pretreatment. Activation of two enzymes, PLCγ1 and PI 3-K, has been shown to be required for PDGF-induced DNA synthesis. These two enzymes associate with tyrosine phosphorylated PDGF receptor (43, 44). Valius and Kaslauskas, using a panel of "add-back" mutants of the PDGF β-receptor, have shown that association of either PLCγ1 or PI 3-K with the PDGF-β receptor in the absence of other signaling molecules is sufficient to elicit a mitogenic response (1). In our study, however, treatment of FSC with IFN-γ did not modify the activation of PLCγ1 or of PI 3-K in response to the subsequent addition of PDGF. These data indicate that the potentiation of the mitogenic action of PDGF by IFN-γ is independent of these two pathways. Stimulation of the PDGF or EGF receptor also results in the activation of Ras, which triggers the activation of a kinase cascade that ultimately phosphorylates and activates MAPK (8). Activated MAPK is responsible for phosphorylation of other regulatory proteins, and activation of transcription factors that result in induction of growth regulatory genes (8). However, IFN-γ pretreatment had no effects on MAPK activity or c-*myc* and c-*fos* oncogene expression.

It appears that the only combination of cytokines that results in enhanced DNA synthesis results in increased STAT1α activation (i.e., IFN-γ followed by PDGF or EGF). Pretreatment of the cells by IL-1α or TNFα followed by PDGF, did not enhance DNA synthesis or activate STAT1α. The mitogenic effect of thrombin in these cells was also not potentiated by IFN-γ pretreatment. Thrombin also did not potentiate the activation of STAT1α in cells pretreated with IFN-γ. These observations of synergistic effect of IFN-γ and growth factors on STAT1α activation strongly suggest its involvement in growth factor–induced mitogenesis.

The experiments carried out with antisense oligonucleotides provide compelling evidence for a role of STAT1α in mediating the effect of IFN-γ on PDGF or EGF-induced DNA synthesis. The reduction in STAT1α levels was associated with a decrease in DNA synthesis in FSC pretreated with antisense oligonucleotides compared to cells pretreated with sense oligonucleotides before the addition of IFN-γ followed by EGF or PDGF (Fig. 8). The effect of antisense oligonucleotides was modest, causing only a partial decrease in DNA synthesis. However, the extent of reduction is consistent with the modest decrease of STAT1α protein levels in response to the same treatment. Indeed, treatment of cells with IFN-γ for 24 h after transfection of the antisense oligonucleotides, conditions identical to those used for DNA synthesis experiments, showed 25% reduction of STAT1α protein levels (see Fig. 8 *C*). These results may reflect the effective intracellular concentration of oligonucleotides achieved with the cationic lipid-mediated approach in these cells. Alternatively, the partial effect of antisense oligonucleotides may be due to the involvement of other pathways in mediating the positive interaction of IFN-γ and PDGF or EGF, possibly at the level of other members of the STAT family.

IFN-γ or PDGF, despite activating partially overlapping signaling pathways, such as STAT1α, also activate transcription of different sets of genes and result in distinct biologic actions. This study shows that the potentiation of mitogenesis in response to growth factors is observed only after at least 24-h pretreatment of the cells with IFN-γ, whereas short-term pretreatment with IFN-γ before addition of the growth factors does not result in potentiation of DNA synthesis, and simultaneous addition of IFN-γ and PDGF or EGF and IFN-γ actually inhibits DNA synthesis. In addition, IFN-γ alone does not stimulate DNA synthesis, despite its ability to activate STAT1α. The mechanisms underlying these observations are not completely clear. IFN-γ phosphorylates STAT1α via activation of the cytoplasmic tyrosine kinases of the Janus family, JAK1 and JAK2 (45). On the other hand, EGF and PDGF receptors have intrinsic tyrosine kinase activity, and EGF receptor has been shown to physically associate and phosphorylate STAT1α (46). It is possible that different activation pathways result in a different fate of the activated STAT1α. Our data indicate that IFN-γ results in increased abundance and prolonged activation of STAT1α, and that subsequent incubation with PDGF or EGF results in further activation. It is possible that after further activation by PDGF or EGF, STAT1α is directed toward a pathway that results in mitogenesis. Conversely, when the cells are incubated simultaneously with IFN-γ and PDGF, or when PDGF is added shortly after IFN-γ, STAT1α follows an IFN-γ–dependent pathway which does not result in mitogenesis, and therefore the mitogenic effect of PDGF is not potentiated. The persistent phosphorylation of STAT1α after treatment with IFN-γ is likely the critical factor underlying its potentiating effect, since IFN-α, which induces only a short-lived STAT1α phosphorylation, did not potentiate DNA synthesis (data not shown).

Silvennoinen et al. (18) have shown that activation of STAT1α is not sufficient to induce mitogenesis, since cells expressing a dominant negative mutant Ras are unable to activate MAPK despite normal activation of STAT1α. The independence of the Ras/MAPK pathway and the STAT1α activation is confirmed by our studies. In fact, activation of STAT1α, as a result of the combination of IFN-γ and PDGF, does not result in potentiation of MAPK activity. Conversely, we find that STAT1α is itself involved in mitogenesis, although our data cannot establish if simultaneous activation of Ras/MAPK or other pathways is required for optimal DNA synthesis. Interestingly, although STAT1α may bind to the SIE of the c-*fos* promoter, mRNA encoding for this protooncogene was not upregulated. As indicated above, this element also binds STAT3 homodimers and STAT1α/STAT3 heterodimers; of note is that STAT3 was not upregulated. Moreover, the serum-response element of the same promoter is $\sim$ 5-fold more potent than the SIE in mediating c-*fos* transcription (29), and the binding activity to this element is regulated via MAPK, which is not altered by IFN-γ. Therefore it is likely that STAT1α acts on a target different from the c-*fos* promoter.

We have shown that the potentiation of the effect of growth factors with IFN-γ is observed in at least three primary cells of mesenchymal origin, including FSC, mesangial cells, and human foreskin fibroblasts. These findings are relevant for understanding the pathophysiology of liver and kidney diseases that lead to fibrosis and organ damage. Proliferation of

these cells is one of the mechanisms that ultimately results in fibrosis and scarring, since the final net increase in extracellular matrix is dependent on increased matrix secretion per cell as well as on the increased number of cells. In vivo investigation has shown that PDGF and PDGF receptor expression is upregulated in liver and kidney diseases (47, 48). Studies using cell culture have indicated that FSC and mesangial cells are sources of PDGF, thus identifying these cells as possible target of an autocrine loop (21, 49). The effect of IFN-γ pretreatment on DNA synthesis was dramatic, since the sequential addition of IFN-γ and PDGF resulted in up to 100-fold increase in DNA synthesis when compared to unstimulated cells. Chronic liver and kidney diseases are characterized by an intense infiltration of activated T lymphocytes, which secrete IFN-γ (50). In addition, transgenic mice overexpressing IFN-γ in the liver, show histologic features of chronic active hepatitis (51). Therefore, it is likely that interaction between IFN-γ and growth factors occurs in vivo during inflammation. As a consequence, the cells would acquire an activated proliferative phenotype, causing a greater accumulation of extracellular matrix. These observations identify STAT1α as a common pathway which may be a potential target for inhibition in vivo, in an attempt to reduce cell proliferation and ameliorate tissue fibrosis.

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