Activation of the ERK1/2 and p38 Mitogen-activated Protein Kinase Pathways Mediates Fibroblast Growth Factor-induced Growth Arrest of Chondrocytes*  

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Fibroblast growth factors (FGFs) regulate long bone development by affecting the proliferation and differentiation of chondrocytes. FGF treatment inhibits the proliferation of chondrocytes both in vitro and in vivo, but the signaling pathways involved have not been clearly identified. In this report we show that both the MEK-ERK1/2 and p38 MAPK pathways, but not phospholipase Cγ or phosphatidylinositol 3-kinase, play a role in FGF-mediated growth arrest of chondrocytes. Chemical inhibitors of the MEK1/2 or the p38 MAPK pathways applied to rat chondrosarcoma (RCS) chondrocytes significantly prevented FGF-induced growth arrest. The retinoblastoma family members p107 and p130 were previously shown to be essential effectors of FGF-induced growth arrest in chondrocytes. The dephosphorylation of p107, one of the earliest events in RCS growth arrest, was significantly blocked by MEK1/2 inhibitors. In contrast, the p38 MAPK inhibitors, whereas that of p130, which occurs later, was partially prevented both by the MEK and p38 inhibitors. Furthermore, by expressing the nerve growth factor (NGF) receptor, TrkA, and the epidermal growth factor (EGF) receptor, ErbB1, in RCS cells we show that NGF treatment of the transfected cells caused growth inhibition, whereas EGF did not. FGF- and NGF-induced growth inhibition is accompanied by a strong and sustained activation of ERK1/2 and p38 MAPK and a decrease of AKT phosphorylation, whereas EGF induces a much more transient activation of p38 and ERK1/2 and increases AKT phosphorylation. These results indicate that inhibition of chondrocyte proliferation by FGF treatment both ERK1/2 and p38 MAPK signaling and also suggest that sustained activation of these pathways is required to achieve growth inhibition.

Fibroblast growth factors (FGFs) are potent regulators of cell proliferation, migration, and differentiation, and it has become evident that the most important role of FGF signaling is during development, including skeletal formation (1–4). Development of most skeletal elements occurs through the multistep process of endochondral ossification, in which a cartilage template is converted into bone. Chondrocytes transit through maturation stages of proliferation, prehypertrophy, and hypertrophy within the epiphyseal growth plate and eventually undergo apoptosis. FGF signaling inhibits endochondral bone growth by regulating the proliferation and differentiation of chondrocytes (4). Gain of function mutations of FGF receptor 3 (FGFR3), which is expressed in growth plate chondrocytes, cause several forms of human dwarfism (4, 5), and conversely, mice lacking FGFR3 showed overgrowth of long bones (6, 7). In line with this genetic evidence, we showed previously (8, 9) that FGF inhibits proliferation of chondrocytes both in vitro and in vivo and that this phenomenon requires STAT1 function. Furthermore, we established that two members of the retinoblastoma (Rb) protein family, p107 and p130, but not pRb, are essential downstream mediators of FGF-induced growth arrest in chondrocytes (10). The function Rb proteins is regulated by phosphorylation (11), and a very early event in the growth arrest of chondrocytes following FGF treatment is the rapid dephosphorylation of p107, which took place even in the absence of new RNA or protein synthesis (12).

FGFs activate a complex network of signaling and transcriptional events in chondrocytes leading to growth arrest (8–10, 12, 13). Although the studies summarized above identified STAT1 and Rb proteins as essential mediators of FGF-induced chondrocyte growth arrest, the signal transduction pathways that utilize STAT1 or lead to dephosphorylation of Rb proteins have not been clearly identified.

FGF binding to its cognate receptors induces receptor tyrosine kinase activation and transphosphorylation of the receptor dimers. The phosphorylated tyrosines function as binding sites for Src homology domain 2 or phosphotyrosine binding domains of a variety of downstream signaling enzymes and adaptor proteins (14, 15). A key component of FGF signaling is the docking protein FRS2, which binds to the FGFs and recruits several signal transducing molecules leading to the activation of the mitogen-activated protein kinase (MAPK) cascade and the PI3K-AKT antiapoptotic pathway (16–18).

The MAPKs are focal points for diverse extracellular stimuli and regulate the activities of kinases or transcription factors downstream, thereby influencing gene expression and cellular responses (19). The MAPK family includes the following: 1) extracellular signal-regulated kinases (ERK1 and ERK2); 2) c-Jun N-terminal or stress-activated protein kinases; and 3) the p38 kinases α, β, γ, and δ (20). In mammalian cells the Ras-Raf-MEK1/2-ERK1/2 cascade is activated by growth factors and is implicated in cell proliferation, differentiation, and survival (21–24). Unlike the ERK signaling pathway, p38 and...
anti-BrdUrd monoclonal antibody (Amersham Biosciences) followed several washes, analysis of BrdUrd incorporation was performed using horseradish peroxidase-conjugated anti-mouse and anti-rabbit second-antibodies. Cell lysates were prepared by lysis (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) in the presence of protease and phosphatase inhibitors (1 mM phenylmethlysulfonyl fluoride, 1 µg/ml aprotinin, leupeptin, and pepstatin, 1 mM sodium orthovanadate, 10 mM sodium fluoride). Lysates were clarified by centrifugation at 10,000 x g for 1 h at 4 °C. Protein concentration was determined by the Bradford methods (Bio-Rad).

**Reagents and Antibodies**—Antibodies against PLCγ1, JNK1, phospho-JNK1/2, ERK2, TrkA, EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-p38 MAPK, phospho-AKT, ART, phospho-p44/42 MAPK, p44/42 MAPK were from Cell Signaling Technology. Anti-phospho-tyrosine 4G10 was from Upstate Biotechnology Inc. The antibody anti-EGFR was a generous gift from Dr. J. Schlessinger (Yale University). The horseradish peroxidase-conjugated anti-mouse- and anti-rabbit secondary antibodies were purchased from Promega Inc. Anti-BrdUrd monoclonal antibody was from Amersham Biosciences (RPN-202). Anti-mouse secondary antibody Cy3-conjugated was from the Jackson Laboratories. The p38 MAPK Assay Kit was from Cell Signaling Technology (catalog number 4820). MEK1/2 inhibitors (PD98059 and U0126) were from Cell Signaling Technology. p38 MAPK inhibitors (SB202190 and SB203580) and PLCγ inhibitor (U73122) were from Calbiochem. Mouse nerve growth factor (NGF) was purchased from Harlan. Human recombinant epidermal growth factor (EGF) was from Intergen. Recombinant human FGF1 and heparin (Sigma, 10 µg/ml) were a gift from Dr. J. Schlessinger (Yale University). The recombinant adenovirus expressing hTrkA (human TrkA) was kindly provided by Dr. J. Schlessinger (Yale University). The pCMV-VSV-G plasmid by calcium phosphate precipitation (30).

**MATERIALS AND METHODS**

**Enzyme-Linked Immunosorbent Assay (ELISA)**—The ELISA was performed as described previously (29). Mean absorbance was calculated as a ratio of BrdUrd-positive nuclei to the total Hoechst-stained nuclei.

**Cloning and Retroviral Infections**—TrkA cDNA was subcloned into pLXSN retroviral expression vector that contains a neomycin resistance gene. The cDNA was cut from cDNA/TrkA expression vector (gift from Dr. M. Chao, New York University), by restriction digestion with EcoRI and cloned into pLXSN generating pLXSN/TrkA. The pLXSN/ErbB1 retroviral expression vector was a gift from Dr. J. Schlessinger (Yale University).

**Cell Culture**—Rat chondrocytoma cells (RCS) were maintained as monolayer cultures in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) as described previously (29). RCS expressing TrkA (RCS/TrkA), RCS expressing EGFR (RCS/ErB1), and 293 gag-pol-expressing cells were cultured under the same conditions.

**Cloning and Retroviral Infections**—TrkA cDNA was subcloned into pLXSN retroviral expression vector that contains a neomycin resistance gene. The cDNA was cut from cDNA/TrkA expression vector (gift from Dr. M. Chao, New York University), by restriction digestion with EcoRI and cloned into pLXSN generating pLXSN/TrkA. The pLXSN/ErB1 retroviral expression vector was a gift from Dr. J. Schlessinger (Yale University).

**293 gag-pol-expressing cells were used for production of retroviruses. Cells were plated at 60% confluency in 100-mm plates and cotransfected with 5 µg of pLXSN/TrkA or pLXSN/ErbB1 plasmids and 5 µg of pCMV-VSV-G plasmid by calcium phosphate precipitation (30). The following day the medium was changed, and 48 h later the viral supernatant was collected. About 2 x 10^5 RCS cells were infected with 1 ml of viral supernatant in the presence of 5 µg/ml Polybrene for 5 h. The cells were selected with genetin G418 for 2 weeks. Protein expression of TrkA or ErbB1 was further analyzed. Pools of retroviral expressing cells were infected with 5 x 10^5 RCS cells in 24-well plates (Costar) for 48 h in DMEM containing 10% FCS. Recombinant human FGF1 and heparin (Sigma, 10 µg/ml), NGF, or EGF were added for 20 h at the indicated concentrations, and the cells were labeled with 1 µM bromodeoxyuridine (BrdUrd; Sigma) for 4 h at 37 °C. The cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature, permeabilized for 10 min with 0.5% Triton in phosphate-buffered saline, and treated for 15 min with 1 x HCl. After several washes, analysis of BrdUrd incorporation was performed using an anti-BrdUrd monoclonal antibody (Amersham Biosciences) followed by anti-mouse secondary antibody conjugated with Cy3 (1:100). Cell nuclei were stained with a solution of 0.5 µg/ml of Hoechst (Sigma) in phosphate-buffered saline for 20 min prior to mounting on slides. The fluorescence was visualized using an Axiosplan 2 Zeiss microscope equipped with a digital camera. The frequency of S phase cells was calculated as a ratio of BrdUrd-positive nuclei to the total Hoechst-stained nuclei.

**Growth Factor Stimulation and Cell Lysate Preparation**—RCS cells were starved overnight in DMEM and stimulated for the indicated times and at the indicated concentrations at 37 °C with FGF1 and heparin, NGF, or EGF in DMEM. After stimulation, cells were washed once with cold phosphate-buffered saline and lysed either in RIPA buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100), or in lysis buffer (HEPES, pH 7.5, 750 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10 mM 4G10), in the presence of protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, leupeptin, and pepstatin, 1 mM sodium orthovanadate, 10 mM sodium fluoride). Lysates were clarified by centrifugation at 13,000 x g for 20 min at 4 °C. Protein concentration was determined by the Bradford methods (Bio-Rad).

**Immunoprecipitation and Western Blot Analysis**—500 µg of protein extract was immunoprecipitated with specific antibodies and 40 µl of Protein A Plus-agarose (Oncogene Science) over night at 4 °C. The immunocomplexes were washed 5 times in lysis buffer and resuspended in Laemmli sample buffer. Immunoprecipitates or total protein extracts (20–30 µg) were boiled separately by SDS-PAGE and electrotransferred on nitrocellulose membranes (Protran®). The membranes were blocked in TBSB (10 mM Tris, pH 7.4, 0.5 mM NaCl, 0.1% Tween 20) containing 3% bovine serum albumin (Sigma) or in TBS containing 5% dried milk. The blots were first probed with the indicated primary antibodies in 3% bovine serum albumin/TBST and then with horseradish peroxidase-conjugated secondary antibodies in TBSB. Proteins were visualized by ECL detection system (Amersham Biosciences).

**RESULTS**

For our experiments we used the RCS cell line that displays numerous chondrocytic characteristics, including expression of type II, type IX, and type XI collagen and Alcian blue-stainable cartilage-specific proteoglycans (29). We treated RCS cells with FGF1, a high affinity ligand for all known FGFR isoforms. In preliminary studies, we confirmed that RCS cells expressed FGFR3 and FGFR2, which are phosphorylated at tyrosine residues upon treatment with FGF1 (not shown). As reported previously (8, 10, 13), FGF1 treatment of RCS resulted in a drastic and dose-dependent growth inhibition, reflected in the reduction of the proportion of cells incorporating BrdUrd. Inhibition of DNA synthesis was rapid and reached a maximum by 16–20 h after treatment.

**FGF Stimulation of RCS Cells Leads to the Activation of ERK1/2, p38 MAPK, and PLCγ but Not JNK and AKT—**To determine which of the signal transduction pathways activated by FGF1 in RCS cells may contribute to the growth-inhibitory
response, we studied the activation of known FGF signaling pathways, including those of the MAPK family, PI3K and PLCγ, in FGF-treated RCS cells.

FGF1 (100 ng/ml) was added to RCS cells for different times, and cellular lysates were analyzed. Fig. 1A shows that FGF1 stimulation led to a strong phosphorylation of ERK1 and ERK2 that was detectable at 5 min and persisted up to 1 h. p38 MAPK was also phosphorylated upon FGF1 stimulation, whereas no activation of JNK1 (p46) and JNK2 (p54) was detected. To verify the phosphorylation of p38 MAPK led to its activation, an in vitro kinase assay was performed. The transcription factor ATF-2 is a direct substrate for p38 MAPK in vitro and in vivo; therefore, its phosphorylation can serve as a measure of p38 activity (31). Fig. 1C shows that FGF1 is able to induce p38-mediated phosphorylation of ATF-2, which peaked at 15 min and decreased to basal levels in 3 h.

The activation of PI3K induced by FGF1 in RCS cells was determined by analyzing the phosphorylation of AKT, a downstream effector of PI3K signaling (32). Immunoblotting using an antibody against the phosphorylation site (Ser-473) in AKT shows that FGF1 did not activate AKT (Fig. 1A). Untreated serum-starved cells exhibit a basal level of AKT phosphorylation that was slightly reduced after 15 min of FGF1 treatment. This result is in contrast with the finding that FGF strongly induces phosphorylation of AKT in fibroblasts (33).

PLCγ is phosphorylated on tyrosine residues following binding to activated FGFRs (34). PLCγ was immunoprecipitated from cellular lysates using an anti-PLCγ antibody followed by a Western blot using an anti-phosphotyrosine antibody. As shown in Fig. 1B, FGF1 strongly activated PLCγ after 5 min of treatment. Thus, these results show that FGF1 stimulation of RCS leads to the activation of ERK1/2, p38 MAPK, and PLCγ but not JNK1/2 and to a decrease of the basal levels of phosphorylated AKT.

MEK1/2 and p38 MAPK Inhibitors Block FGF-mediated Growth Arrest of RCS Cells—We used specific chemical inhibitors for ERK1/2, p38 MAPK, and PLCγ to determine their effect on FGF-induced growth arrest. The compounds used were the MEK1/2 inhibitors, U0126, PD98059, SB202190, and SB203580, which inhibit p38α and p38γ MAPK, and the PLCγ inhibitor U73122. The MEK1/2 inhibitors selectively prevent the activation of MEK1/2 by Raf with a subsequent blocking of the ERK1/2 cascade. U0126 was shown to be able to inhibit both MEK1 and MEK2, whereas PD98059 inhibits MEK1 more potently than MEK2. SB202190 and SB203580 are able to block the kinase activity of p38α and p38γ MAP kinases, and U73122 has been shown to inhibit PLCγ-associated phosphatidylinositol 4,5-bisphosphate hydrolysis (35–37).

The effect of MEK1/2 inhibitors on ERK1/2 activation and on RCS proliferation was tested. Treatment of RCS cells with PD98059 and U0126 produced a significant reduction in FGF1-induced ERK1/2 activation. A complete block of ERK1/2 phosphorylation was seen using 100 μM PD98059 and 20 μM U0126 (Fig. 2A and data not shown). These concentrations of the inhibitors were able to partially counteract the FGF-mediated growth arrest of RCS cells. BrdU incorporation in the presence of the inhibitors shows that the effect was detectable at concentrations of FGF1 ranging from 0.5 to 10 ng/ml (Fig. 2A).

In order to determine whether p38α and p38γ MAPKs also played a role in the response to FGF, we used two different concentrations of SB202190 and SB203580 (10 and 30 μM), which inhibited p38 by the effect on its target MAPKAP kinase-2 activity (data not shown) (38, 39). Both inhibitors were very effective at blocking the FGF1-mediated growth arrest with a maximal effect occurring at 30 μM (Fig. 2B). On the other hand, inhibition of PLCγ activity in the FGF-treated RCS cells using U73122, at concentrations of 1 and 5 μM, did not reverse the FGF-induced growth arrest (Fig. 2C). Inhibition of PI3K activity by wortmannin also had no effect on FGF-mediated growth arrest (data not shown). Thus, these results indicate that p38 MAPK and ERK1/2 signaling play a role in FGF-mediated growth arrest of RCS cells.

The MEK1/2 Inhibitors Affect p38 MAPK Activity—Recent studies (40) have shown that cross-talk between signaling cascades can modulate the response to growth factors. To determine whether inhibition of MEK1/2 could affect the activity of p38 and vice versa, we studied the phosphorylation pattern of ERK1/2, and the enzymatic activity of p38 MAPK in the absence and presence of a MEK1/2 inhibitor (U0126) and a p38 MAPK inhibitor (SB202190). As expected, FGF1-induced ERK1/2 phosphorylation was blocked by 20 μM U0126 but not by 20 μM SB202190, indicating that p38 MAPK is not required for ERK1/2 activation (Fig. 3A). We determined the effect of U0126 on p38 activity by an in vitro kinase assay using ATF-2 as substrate. Fig. 3B shows that the p38 activity induced by
FGF1 was reduced in the presence of the MEK1/2 inhibitor, indicating that MEK1/2-ERK1/2 signaling can contribute to p38 MAPK activation or that the U0126 compound is not completely specific. Similar results were obtained with the PD98059 MEK1/2 inhibitor (data not shown). This result suggests that by using our experimental conditions, the ability of MEK1/2 inhibitors to prevent the FGF-induced growth arrest may depend in part on their effect on p38 MAPK activity.

The ERK1/2 and p38 MAPK Pathways Are Involved in p107 and p130 Dephosphorylation

Laplantine et al. (10) have shown that FGF treatment of RCS cells causes dephosphorylation of Rb family proteins and that p107 and p130 function is required for FGF-induced growth inhibition of chondrocytes in vitro and in vivo. Further data (12) indicated that dephosphorylation of p107 and p130 is generated by different mechanisms and that p107 dephosphorylation is one of the earliest events in FGF-induced growth arrest of chondrocytes. We have studied the effect of the MEK1/2 and p38 MAPK inhibitors on the dephosphorylation of p107 and p130 induced by FGF in RCS cells. As shown in Fig. 4A, RCS cells contain a mixture of hyper- and hypophosphorylated p107 molecules that upon FGF treatment are rapidly converted to the hypophosphorylated form. This event is significantly prevented by the U0126 MEK1/2 inhibitor but not by the p38 inhibitor SB202190. The effect of the MEK1/2 inhibitor is clearly detectable at 1 h after FGF addition and persists up to 20 h. On the other hand, growing RCS cells contain predominantly hyperphosphorylated forms of p130, and p130 dephosphorylation is clearly detectable only after several hours of treatment with FGF1. The ERK1/2 and p38 MAPK pathways affect FGF-induced p107 and p130 dephosphorylation in a distinct way. ERK1/2 signaling is required for p107 dephosphorylation,
MAPK Signals in FGF-induced Chondrocyte Growth Arrest

Expression of TrkA and ErbB1 in RCS Cells—To better understand the role of p38 MAPK and ERK1/2 in the response to FGF of RCS cells, we created RCS cells expressing the NGF receptor TrkA or the EGF receptor ErbB1, two tyrosine kinase receptors that are known to activate both pathways. Signaling through these two receptors has been shown to elicit different responses in PC12 cells where EGF stimulation induces cellular proliferation, whereas NGF and FGF stimulation results in differentiation into a sympathetic neuron-like phenotype (41, 43). To compare tyrosine kinase receptor signaling in an inhibitory versus non-inhibitory response, the signal transduction pathways activated by EGF in RCS/ErbB1 cells were investigated. Interestingly, the addition of EGF (100 ng/ml) leads to a transient ERK1/2 and p38 MAPK activation compared with FGF1 and NGF. Although a strong ERK1/2 phosphorylation was detected after 1 min of stimulation, it declined to lower levels after 15 min (Fig. 7A). p38 MAPK activity reached its maximum in 15 min and declined to basal levels within 1 h (Fig. 7B). EGF was unable to activate JNK1/2 in RCS/ErbB1 cells but caused AKT phosphorylation (Fig. 7A). Together these results show that unlike FGF1 and NGF, EGF signaling does not inhibit RCS proliferation and only transiently activates ERK1/2, p38 MAPK, while increasing phosphorylation of AKT.

Time Course of ERK1/2 and p38 MAPK Activation—It has been shown (48) that both the magnitude and duration of ERK1/2 activation can determine the nature of the cellular response to growth factors. In PC12 cells, EGF transiently stimulates ERK1/2 and p38 MAPK and induces cellular proliferation. In contrast, NGF and FGF cause sustained activation of both pathways and neuronal differentiation (43, 49). To compare the kinetics and magnitude of ERK1/2 activation in response to FGF1, NGF, and EGF stimulation of RCS/TrkA and RCS/ErbB1 cells, ERK1/2 phosphorylation was monitored at various time points up to 5 h. As shown in Fig. 8, FGF1 induced a strong and prolonged ERK1/2 activation that peaked in 5 min and persisted up to 5 h. NGF stimulation of RCS/TrkA also led to a strong and prolonged ERK1/2 activation that peaked in 5 min and was still detectable after 5 h of stimulation. However, EGF stimulation of RCS/ErbB1 led to a very rapid but transient phosphorylation of ERK1/2 that peaked at 1 min and decreased to the basal level by 3 h.

As shown in Figs. 1C, 6B, and 7B, the activity of p38 MAPK upon FGF1 and NGF stimulation is stronger, reaches its max-
Fig. 5. Response of RCS cells expressing TrkA or ErbB1 receptors to NGF or EGF. A and B, expression and activation of TrkA and ErbB1 receptors in RCS cells. Parental RCS cells as well as cells stably expressing the TrkA (RCS/TrkA) or ErbB1 (RCS/ErbB1) receptors were serum-starved overnight and treated with 100 ng/ml NGF or 100 ng/ml EGF for 5 min, respectively. The expression of the receptors was tested by immunoprecipitation (IP) with anti-TrkA or anti-ErbB1 antibody followed by immunoblotting as indicated. The activation of receptors was detected using the anti-phosphotyrosine antibody 4G10 (P-Tyr). C and D, NGF inhibits proliferation of RCS/TrkA cells, whereas EGF does not inhibit proliferation of RCS/ErbB1 cells. Cells were seeded on coverslips at a density of 2 × 10^4 cells/well (24-well plates) in DMEM containing 10% FCS. After 24 h, the medium was replaced, and the cells were treated with indicated concentrations of NGF or EGF. Cells were labeled with BrdU (5 μg/ml) for the last 4 h, fixed, and processed for immunofluorescent detection of BrdU.
maximum at 15 min, and is still high after 1 h, while upon EGF stimulation, p38 activity reaches its maximum at 15 min but then decreases to basal levels. Thus a clear correlation between the magnitude and particularly the duration of ERK1/2 and p38 MAPK activation and growth arrest can be detected in RCS cells upon stimulation of the NGF, EGF, or of the endogenous FGF receptors. NGF and FGF receptor stimulation leads to sustained activation of p38 MAPK and ERK1/2 and to growth arrest, although stimulation of the EGF receptor does not cause growth arrest and is accompanied by a significant but short-lived activation of p38 MAPK and ERK1/2.

**DISCUSSION**

The rate of proliferation and differentiation of chondrocytes determines the overall growth in the length and shape of long bones. Evidence from human and mouse genetics indicates that longitudinal bone growth is negatively regulated by FGF signaling (4), and accordingly, FGF signaling inhibits chondrocytes proliferation in *vitro* and *in vivo* (8), an unusual response for FGFs that usually act as mitogens in other cell types. Although genetic evidence clearly identified STAT1 as an essential mediator of the FGF-induced growth arrest of chondrocytes both in *vitro* and *in vivo* (8, 9), the mechanism by which STAT1 participates in this process is still unclear and may not require that it function as a transcriptional regulator. Indeed data from our laboratory2 show that interferon γ, a potent inducer of STAT1 transcriptional activation, does not induce

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2 A. Raucci, E. Laplantine, and C. Basilico, unpublished data.
growth arrest in RCS cells, indicating that either STAT1 function is necessary but not sufficient to promote growth arrest or that its phosphorylation is not required. In this report we show that two components of the MAPK family, ERK1/2 and p38 MAPK, also play an important role in FGF-mediated growth arrest in chondrocytes.

**Blocking the ERK or p38 MAPK Pathways Prevents the Growth Arrest Induced by FGF in Chondrocytes**—We have used the RCS cell line to show that FGF causes a strong and prolonged activation of the ERK1/2 and p38 MAPK pathways in chondrocytes. By exposing RCS cells to specific inhibitors of MEK-ERK1/2 or p38 signaling, we showed that inhibition of the function or the activation of these enzymes partially or completely prevented FGF-induced growth arrest, in that cells continued to proliferate and enter the S phase similarly to untreated controls. On the other hand, inhibition of PLCγ activity had no significant effect. Thus PLCγ does not appear to play an important role in FGF-mediated growth inhibition, similar to what had been shown previously (50) for FGF induction of proliferation in fibroblasts.

Interestingly, inhibition of ERK1/2 or p38 MAPK differentially affects the FGF-mediated growth arrest of RCS. Whereas SB202190 and SB203580 (p38 MAPK inhibitors) almost completely restore cell proliferation, PD98059 and U0126 (MEK1/2 inhibitors) cause a more partial block of the FGF effect. We have also shown that the p38 MAPK inhibitor, SB202190, does not influence the activation of ERK1/2 by FGF1, whereas the MEK1/2 inhibitor, U0126, partially blocks the p38 MAPK activity (detected by phosphorylation of the substrate ATF-2).

Together with the observation that the ability of p38 MAPK inhibitors to prevent the FGF-induced growth arrest is stronger than that of the ERK inhibitors, this result could be interpreted to suggest that the effect of the ERK inhibitors depends at least in part on the inhibition of the p38 MAPK pathway. Although U0126 has generally been considered specific for MEK1/2, it is possible that at the concentrations used, it could also directly affect p38 MAPK activity or activation (36, 37). Alternatively, it is possible that the MEK1/2-ERK1/2 pathway contributes to p38 MAPK activation, as suggested by Morooka and Nishida (49). We have found that the phosphorylation of p38 MAPK induced by FGF in RCS cells is partially inhibited in the presence of the U0126 compound (data not shown). We have attempted to investigate this point further by using dominant-negative forms of MEK1 and ERK2, but we found that in our system the inhibition of ERK signaling obtained by the expression of these mutants was very slight, and thus the results obtained are not conclusive. However, irrespective of the mechanism by which the MEK1/2 inhibitor may affect p38 MAPK activity, the data on p107 and p130 dephosphorylation indicate that these two pathways produce signals differentially affecting the ability of FGFs to induce dephosphorylation of Rb proteins. ERK1/2 activity appears to be important for p107 dephosphorylation, which is not affected by the p38 MAPK inhibitors, whereas p130 dephosphorylation is reduced by inhibition of both pathways. Other FGF responses are also differentially sensitive to inhibition of p38 MAPK or ERK1/2 signaling. We have shown previously that FGF treatment of RCS induces the expression of several differentiation-related genes, such as osteopontin, MMP13, and TIMP1 (12). This induction is completely blocked by the MEK1/2 inhibitors and not by the p38 inhibitors. Thus, although the effect of the MEK1/2 inhibitors could depend in part on inhibition of p38 MAPK activity, this cannot be the sole mechanism by which these compounds prevent many FGF responses in RCS cells.

ERK1/2 are usually activated by growth factors and are implicated in cell proliferation, differentiation, and survival (22–24), whereas p38 MAPK is preferentially activated by inflammatory cytokines, cellular stresses, withdrawal of growth factors, and proapoptotic stimuli (25). It is interesting to note that two recent reports (51, 52) on the growth arrest caused by stress responses have detected a rapid dephosphorylation of p107 in response to UV or oxidative stress stimuli, which are known to cause strong activation of p38 MAPK (53). In our case, however, the rapid dephosphorylation of p107 induced by FGF in chondrocytes appears to depend on the activation of the ERK pathway, rather than on p38 MAPK. We had suggested previously that the rapid dephosphorylation of p107 induced by FGF in RCS cells, which occurs in the absence of new protein synthesis, could be due to "activation" of a specific phosphatase (10, 12, 13). In view of the results presented in this report, it is likely that activation of this phosphatase, which may be due to phosphorylation, requires the direct or indirect action of ERK1/2 but that the p38 MAPK may also contribute to its activation.

In contrast to p107, p130 dephosphorylation is a late event that we had defined as a characteristic of the "maintenance" of growth arrest and probably results from inactivation of cyclin-dependent kinases (12). p130 dephosphorylation is partially blocked by both types of inhibitors. ERK1/2 and p38 MAPK can regulate the expression and the stability of cyclin-dependent kinase inhibitors such as p21 (54–57), which is induced by FGF in RCS cells. A block in p21 induction could affect p130 dephosphorylation. Together with previous experiments aimed at identifying the key molecules and events that determine the growth-inhibitory response of chondrocytes to FGF signaling, the data presented here highlight the concept that in these cells FGF induces a complex network of signaling and transcriptional events, each of which is necessary, but not sufficient, to establish a growth-inhibitory response.

**The Duration of MAP Kinase Activation Correlates with the Biological Response of RCS Cells to Growth Factors**—We created new RCS cell lines expressing two different tyrosine kinase receptors, TrkA and ErbB1, the receptors for NGF and EGF, respectively. Stimulation with NGF of RCS cells expressing TrkA induces growth inhibition and G1 arrest, whereas stimulation of RCS cells expressing ErbB1 with EGF does not. In both cases the receptors are activated upon ligand binding. Notably, like FGF, NGF induces sustained activation of ERK1/2 and p38 MAPK, and ERK1/2 and p38 MAPK kinase inhibitors prevent the NGF-mediated growth arrest. In contrast, EGF induces a transient activation of ERK1/2 and p38 MAPK. These findings are in accordance with numerous reports indicating that the duration of MAPKs signal may hold the key to very different cellular outcomes.

In PC12 cells, NGF and FGF induce sustained activation of MAPKs, which is required for cell cycle arrest and terminal differentiation, whereas EGF, which only elicits a transient activation of MAPKs, does not promote differentiation but induces proliferation (43, 49). In addition, overexpression of EGFR in PC12 cells leads to a prolonged MAPK response resulting in cell differentiation (58). Sustained activation of MAPK has been associated with nuclear accumulation of these enzymes (58) where it may modulate gene expression via the phosphorylation of transcription factors, so that the quantitative differences in the duration of MAPK activation may be reflected in qualitative changes in gene expression. For example, the activation of MAPKs regulates AP-1 activity through the phosphorylation of distinct substrates (59, 60). The AP-1 complexes, which include the c-Jun and c-Fos family, regulate the transcription of a number of genes, and depending on the

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3 R. Priore and A. Raucci, unpublished data.
dimer combination they can be either positive or negative regulators of gene expression (61). It has been demonstrated that Fra-1, Fra-2, c-Jun, and JunB are substrates of sustained ERK1/2 activity (62), and we have shown previously that FGF treatment causes a strong up-regulation of the expression of Fra-1, c-Jun, and JunB in RCS cells (12). Thus different modifications of the activity of proteins that make up the AP1 complex due to sustained or transient MAPK activation could result in different programs of gene expression that play a role in directing the response of RCS cells toward growth-inhibitory or growth-stimulating pathways.

The mechanisms by which these different tyrosine kinase receptors induce distinct patterns of MAPK activation and distinct biological responses in the same cell type are still unclear. Considerable attention has been focused on the signaling molecules linking the receptors to the ERK1/2 pathway. The ErbB1, TrkA, and FGF receptors all recruit a variety of signaling molecules upon growth factor stimulation. Most of these effectors are shared by the three receptors including PLCγ, Shp-2, Shc, and Grb2 (15), but TrkA and FGFRs associate with an additional signaling molecule, FRS2 (16, 63). Activated FRS2 has been shown to form a multiprotein complex with Shp2 and Grb2, which has been postulated to play an important role in the sustained activation of ERK1/2 elicited by NGF and FGF in PC12 cells (16, 17).

**The PI3K/AKT Pathway**—In contrast with finding that FGF protects several types of cells from apoptosis by activating the anti-apoptotic AKT pathway, FGF promotes apoptosis in chondrocytes (9). We show here that FGF1 does not activate AKT in RCS cells, and the basal level of AKT phosphorylation does not susceptible to the growth-arresting effect of NGF (67). Thus the inhibition of proliferation that FGFs cause in chondrocytes should shed light on the complex interplay between the genes and their products that are responsible for growth arrest and initiation of differentiation induced by FGF in chondrocytes, and on how the expression or the activity of these genes is regulated by the MAPKs cascades.

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