The 10T1/2-MRF4 fibroblast/myogenic cell system was used to address the following interrelated questions: whether distinct signaling pathways underlie myogenic inhibition by basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-β; which of these pathways also up-regulates the fibroblast intermediate conductance calcium-activated potassium channel, FIK, a positive regulator of cell proliferation; and whether FIK up-regulation underlies some or all myogenic inhibitory signaling events. The results show that myogenic inhibition in 10T1/2-MRF4 cells, by both bFGF and TGF-β, requires activation of the Ras/mitogen-activated protein (MAP) kinase/MAP kinase-ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, and resultant FIK up-regulation. We show that FIK is instrumental in MEK-dependent suppression of acetylcholine receptor channel expression but that MEK activation and FIK up-regulation are not essential to suppression of myosin heavy chain and myotube formation. These data indicate that Ras/MEK/ERK induction of FIK is pivotal to regulation of certain myogenic events by both receptor tyrosine kinases and TGF-β receptor, and this is also the first demonstration of chronic FIK up-regulation by the TGF-β receptor family. Furthermore, the results define the physiologic signaling requirements for growth factor-stimulated FIK up-regulation, whereas previous work has concentrated on constitutive FIK up-regulation in cells stably transfected with oncprotein signaling molecules. This study, together with earlier work showing that FIK positively regulates cell proliferation, establishes this member of the IK channel family as a multifunctional, growth factor-regulated signaling molecule.

Growth factor stimulation plays a pivotal role in a number of regulated cell growth processes, including the expression of ion channels (1), and gene products that promote or inhibit myoblast growth and terminal differentiation (2). Growth factor removal initiates the events of myogenic differentiation, including withdrawal of proliferating myoblasts from the cell cycle, induced expression of muscle-specific gene products, and the subsequent fusion of myoblasts into multinucleate myofibers. This process has been modeled in the multipotent 10T1/2-MRF4 fibroblast cell system. These cells overexpress the muscle-specific regulatory transcription factor MRF4,7 which is activated upon growth factor withdrawal to produce a terminal muscle phenotype (3). One growth factor in particular, bFGF, is a potent inhibitor of MRF4-mediated myogenesis, although the exact signaling requirements for this action have not been elucidated.

We have shown that another significant physiological action of bFGF in 10T1/2-MRF4 cells is to stimulate the expression of an intermediate conductance calcium-activated potassium channel, FIK. FIK in turn modulates and specifically controls proliferation and differentiation in this and other cell types (4, 5). Thus FIK may be a critical component of growth factor signaling pathways that regulate proliferation and differentiation in skeletal muscle myoblasts. The signaling requirements for growth factor-stimulated FIK up-regulation are also not known, although FIK is constitutively up-regulated in response to Ras or Raf oncprotein expression (6), strongly hinting at a role for the Ras/MEK/ERK system in growth factor modulation of FIK. bFGF signaling via Ras has also been proposed to suppress myogenesis (7–9); therefore, these two lines of work suggest that growth factor-stimulated FIK up-regulation depends on Ras/MEK/ERK activation and that Ras/MEK/ERK-dependent up-regulation of FIK is a general mechanism for myogenic suppression by receptor tyrosine kinase ligands typified by bFGF.

Like bFGF, transforming growth factor-β (TGF-β) is known to activate multiple signaling paths including the Ras/MEK/ERK and p38 MAP kinase pathways, and it regulates a multitude of cell biological functions (10–13). TGF-β also acts as a potent inhibitor of myoblast differentiation in the BC3H1, C2C12, and L6 myoblast cell lines (10, 14–16), although the signaling paths underlying these actions of TGF-β have not been identified. We asked if MEK-dependent up-regulation of FIK could also explain TGF-β-mediated myogenic inhibition in 10T1/2-MRF4 cells. A positive result would further validate a general role for Ras/MEK/ERK-dependent up-regulation of FIK in growth factor-mediated myogenic suppression. In addition, the results would broaden our understanding of which receptor families and which signaling events can regulate the IK channel family.

Experimental Procedures

Cell Culture and Preparation—Experiments were performed using C3H10T1/2 cells constitutively overexpressing a rat MRF4 cDNA (3). 10T1/2-MRF4 cultures were grown on gelatin-coated dishes as undifferentiated myoblasts in serum-replete medium (basal medium Eagle, 

The abbreviations used are: MRF4, muscle regulatory transcription factor 4; bFGF, basic fibroblast growth factor; FIK, fibroblast intermediate conductance calcium-activated potassium channel; MAP, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MHC, myosin heavy chain; MPP, MAP kinase/ERK kinase; TGF-β, transforming growth factor-β; DMEM, Dulbecco’s modified Eagle’s medium; HS, horse serum; EGF, epidermal growth factor; ChTX, charybdotoxin; ACh, acetylcholine; MHC, myosin heavy chain.
15% fetal bovine serum), or they were differentiated via serum withdrawal as described previously (4). For electrophysiology cells were seeded onto 35-mm dishes and grown to confluence in serum-replete medium and then switched to low glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2% horse serum (HS). Growth factors, bFGF, or epidermal growth factor (EGF) (20 ng/ml), TGF-β (5 ng/ml), the FIK blocker charybdotoxin (ChTX), or the p38, or MEK inhibitors SB202190 (10 μM) or PD98059 (25 μM) were then added as noted. For proliferation experiments cells were seeded at 5 × 10^4/well (24-well plates) in low glucose DMEM supplemented with 2% HS for 5 h. This was followed by addition of the appropriate growth factor with or without PD98059 to the media. Cells were treated and counted daily using a hemocytometer, and trypan blue exclusion was used to ensure counts of only viable cells. All cultures were maintained in a humidified, 5% CO₂ atmosphere at 37 °C.

**Immunocytochemistry**—Cells were grown to confluence as described above and switched to low glucose DMEM supplemented with 2% HS and stimulated with bFGF, EGF, or TGF-β (with or without 200 nm ChTX, 25 μM PD98058, or 10 μM SB202190) for 48 h. Cells were rinsed in phosphate-buffered saline (in mM: 150 NaCl, 1.9 NaH₂PO₄·H₂O, and 8.1 Na₂HPO₄), pH 7.3, fixed with a 90% methanol solution and then incubated with the anti-myosin mouse monoclonal antibody MF-20 as described previously (5). Myosin staining was compared with that of control cells (2% HS) for which staining was set to a value of 100. All cells were photographed under bright-field at ×40 magnification.

**Solutions and Reagents**—The standard bath solution used for recording whole-cell FIK channel and acetylcholine (ACh) receptor channel currents contained (in mM) 138 NaCl, 9 KCl, 1 MgCl₂, 1 CaCl₂, and 10 HEPES. The patch pipette solution for these recordings contained (in mM) 150 KCl, 1 MgCl₂, 10 HEPES, and 0.1 EGTA. A23187 (Sigma), bFGF, EGF, TGF-β (Austral Biologicals), ChTX (BACHEM Bioscience), PD98059 (New England Bio Labs), and SB202190 (Upstate Biotechnology, Inc.) were stored as frozen stocks, and aliquots of each were diluted to final concentrations on the day of use. Acute application of compounds to cells during patch clamp recording was accomplished via pressure ejection of solution from blunt-tipped pipettes. All solutions were at pH 7.3, and electrophysiology and immunocytochemistry experiments were done at room temperature, 22–25 °C.

**Electrophysiology**—Patch clamp apparatus, techniques, and cell preparation were as described previously (4, 5). Whole-cell FIK currents were measured during voltage steps to 0 mV (from −70 mV) while intracellular calcium levels were increased by extracellular application of A23187 (1 μM). Whole-cell ACh receptor currents were recorded at −70 mV in response to extracellular application of 100 μM ACh. The analog compensation circuitry of the patch clamp amplifier was used to estimate whole-cell capacitances (expressed in picofarads). Whole-cell currents were normalized to cell capacitance, an indirect measure of membrane area, and expressed as mean ± S.E. current densities in picoamperes/picofarads.

**RESULTS**

bFGF, TGF-β, and EGF Up-regulation of the Calcium-activated Potassium Channel, FIK, Requires Signaling via Ras/MEK/ERK but Not the p38 MAP Kinase Pathway—bFGF up-regulates FIK in 10T1/2-MRF4 cells (4), and increased FIK channel activity stimulates proliferation and suppresses myogenesis. To date, additional growth factor-activated signaling systems that modulate FIK, and thus may regulate muscle cell growth and differentiation, have not been identified. Like bFGF, TGF-β also acts as a potent inhibitor of myogenic differentiation, although it has not been tested on 10T1/2-MRF4 cells. TGF-β activates multiple signaling paths including the Ras/MEK/ERK and p38 signaling cascades, but it is not known which of these downstream pathways contribute to TGF-β-mediated myogenic inhibition. To address these issues we first examined if TGF-β could up-regulate FIK and, if so, by which signaling cascade. This information would then allow us to look at the possible involvement of FIK in TGF-β-induced myogenic suppression. We also looked at FIK and growth regulation in response to EGF, because of the distinctly different actions of this Ras/MEK/ERK activator versus those of bFGF. In 10T1/2-MRF4 and NIH3T3 cells, bFGF up-regulation of FIK persists for the duration of peptide exposure. EGF, however, only transiently up-regulates FIK in 3T3 cells, and it is an inefficient mitogen (6). We hypothesized, therefore, that EGF would also inefficiently stimulate FIK and the proliferative capacity of 10T1/2-MRF4 cells and for these reasons would fail to suppress myogenesis.

FIK channel levels were compared for 10T1/2-MRF4 cells stimulated with TGF-β, EGF, or bFGF. Fig. 1A shows that 24-h TGF-β up-regulated FIK. Mean FIK current density in TGF-β-treated cells (13.5 ± 1.9 pA/pF, n = 18) was greater than the density observed in non-treated controls (2% HS) (6.8 ± 2.4 pA/pF, n = 12) (4) but much less than the density in bFGF-stimulated cells (22.9 ± 2.5 pA/pF, n = 10). TGF-β also failed to stimulate 10T1/2-MRF4 cell growth (Fig. 1B). EGF poorly up-regulated FIK and, as predicted, failed to stimulate growth in 10T1/2-MRF4 cells. Fig. 1A shows that 24-h EGF-treated cells had significantly lower FIK current densities (5.7 ± 2.5 pA/pF, n = 7) than cells stimulated with either bFGF or TGF-β. 48-h EGF exposure gave a FIK density of 4.8 ± 2.3 pA/pF (n = 8). Thus chronic EGF application does not elevate FIK levels beyond those observed in the absence of growth factor treatment.

**Fig. 1. Effects of growth factor-activated Ras/MEK/ERK or p38 signaling on cell growth control and FIK channel expression in 10T1/2-MRF4 cells.** A, whole-cell FIK current densities from 24-h growth factor-stimulated cells (bFGF, EGF, or TGF-β) with or without PD98059 or SB202190. PD98059 decreased bFGF and TGF-β-induced FIK channel functional expression. N.D. indicates not determined. B, growth experiments from cells treated daily with EGF, TGF-β, or bFGF with or without PD98059. Controls received 2% HS only. Results represent data from duplicate experiments. Both chronic EGF and TGF-β treatment fail to stimulate cell growth, and the MEK inhibitor PD98059 inhibits bFGF-stimulated proliferation. C, whole-cell FIK current traces from TGF-β-stimulated cells with or without PD98059. Outward currents recorded at 0 mV in response to extracellular application of 1 μM A23187 have been shown to be due solely to the FIK channel (4).
Since bFGF and TGF-β activate both Ras/MEK/ERK and p38 MAP kinase signaling pathways, we next asked if these pathways are also involved in growth factor up-regulation of FIK, as this would also suggest that they contribute to myogenic suppression. FIK current densities were determined for 24-h growth factor-stimulated 10T1/2-MRF4 cells, with or without the MEK inhibitor PD98059 (25 μM) or the p38 MAP kinase inhibitor SB202190 (10 μM and 25 μM). Fig. 1A shows that for all growth factor treatments the MEK inhibitor PD98059 significantly decreased or completely inhibited FIK up-regulation (0.0 ± 0.0 pA/pF, n = 10, EGF; 4.8 ± 1.0 pA/pF, n = 8 TGF-β; 2.4 ± 0.8 pA/pF, n = 9, bFGF). FIK current traces from cells stimulated with TGF-β with or without PD98059 also clearly reveal that the MEK inhibitor blocks FIK up-regulation (Fig. 1C). As predicted, PD98059 also inhibited proliferation of bFGF-stimulated cells, and the measured growth rate was less than in EGF- or TGF-β-stimulated or in non-treated control cells (Fig. 1B).

Interestingly, the p38 inhibitor SB202190 had no effect on FIK currents measured from cells stimulated with either bFGF (23.5 ± 1.7 pA/pF, n = 10, 10 μM or 20.8 ± 4.0 pA/pF, n = 16, 25 μM) or TGF-β (13.0 ± 1.8 pA/pF, n = 14, 10 μM or 9.7 ± 2.5 pA/pF, n = 11, 25 μM), suggesting that FIK up-regulation is not dependent on the p38 pathway. Our previous work showed that expression of oncogenic Ras constitutively up-regulates FIK in a Raf-dependent manner (6), but that work did not directly address a role for MEK nor did it address the basis for FIK up-regulation in the context of growth factor (rather than oncogene)-mediated signaling. We can now conclude that growth factor up-regulation of FIK requires signaling through Ras/MEK/ERK and not p38 MAP kinase. Furthermore, these results suggest that activation of Ras/MEK/ERK is necessary for 10T1/2-MRF4 proliferation. The failure of EGF and TGF-β to stimulate cell growth may reflect less robust activation of this pathway, relative to bFGF-mediated activation, and therefore less FIK channel up-regulation. However, additional anti-proliferative signaling actions of TGF-β cannot be completely ruled out at this time. These results provide the first indication that the TGF-β receptor family up-regulates FIK and that TGF-β or bFGF activation of Ras/MEK/ERK share a common regulatory function, induction of the FIK channel.

bFGF and TGF-β Suppress Myogenesis via Ras/MEK/ERK-dependent FIK Up-regulation—Having shown that TGF-β up-regulates FIK, we asked if TGF-β also suppresses myogenesis in the 10T1/2-MRF4 system, as it does in other muscle cell lines. To test for myogenic inhibition, 10T1/2-MRF4 cells were switched to differentiating medium (2% HS) with or without TGF-β for 48 h and then assayed for expression of ACh receptor channels and MHC. Whole-cell patch clamp recordings and immunocytochemistry were used to assay for ACh receptor currents and myosin expression, respectively. As shown in Fig. 2, only 7% of TGF-β-stimulated cells expressed MHC. Similarly, the ACh receptor channel current density was significantly lower (4.0 ± 2.7 pA/pF, n = 10) in TGF-β-treated cells than compared with control cells (131.7 ± 22.8 pA/pF, n = 25) (Fig. 2). These data parallel the results obtained with bFGF-treated cells in which only 3% of the cells express MHC, and a complete absence of ACh receptor channel expression is observed (0.0 ± 0.0 pA/pF, n = 6) (Fig. 3). Additionally, bFGF- or TGF-β-treated cells exhibited no evidence of myotube formation (Figs. 2 and 3), suggesting that both bFGF and TGF-β act as potent myogenic suppressors in 10T1/2-MRF4 cells.

We next asked if blocking FIK channel activity could override myogenic suppression induced by TGF-β and evoke an MRF4-dependent ACh receptor channel and MHC expression pattern. TGF-β suppression of ACh receptor expression was overcome by addition of 200 nM ChTX to the growth medium (Fig. 2). The average ACh current density for cells treated with TGF-β plus ChTX (123.8 ± 25.2 pA/pF, n = 18) was significantly greater than the density for cells treated with TGF-β alone (4.0 ± 2.7 pA/pF, n = 10). This value for TGF-β plus ChTX was comparable to the density observed in cells treated 24 h with EGF plus ChTX (171.6 ± 31.5 pA/pF, n = 32) or with bFGF plus ChTX (176.2 ± 49.6 pA/pF, n = 4) (4). Interestingly, TGF-β-stimulated cells treated with ChTX failed to exhibit significant MHC expression (5%) or formation of multinucleate muscle fibers, suggesting that FIK up-regulation suppresses only certain myogenic events.

The ability of PD98059 to inhibit bFGF- and TGF-β-stimulated FIK up-regulation and bFGF-stimulated cell growth (Fig. 1, A and B) suggests a role for Ras/MEK/ERK in these processes. In order to test this, we used PD98059 to ask if MEK activation was also essential to growth factor-mediated myogenic inhibition. A positive result would again suggest that bFGF and TGF-β share a common signaling pathway (i.e. Ras/MEK/ERK) to up-regulate FIK and suppress differentiation in 10T1/2-MRF4 cells. Fig. 3 shows that PD98059 treatment of bFGF-stimulated cells reversed bFGF inhibition of ACh receptor channel expression, resulting in a mean ACh current density (142.6 ± 41.7 pA/pF, n = 10) comparable to that of non-treated control cells (131.7 ± 22.8 pA/pF, n = 25). The level of
Myogenic Inhibition via RAS/MEK/ERK and FIK

Ach receptor currents also increased with PD98059 treatment of TGF-β-stimulated cells (168.4 ± 55.3 pA/pF, n = 9) (Fig. 2). However, PD98059 treatment of bFGF- or TGF-β-stimulated cells failed to evoke MHC expression (8 and 5%, respectively) or multinucleate muscle fiber formation (Figs. 2 and 3). These data suggest that functional FIK channel expression underlies growth factor inhibition of muscle differentiation, as measured by Ach receptor expression in 10T1/2-MRF4 cells. Furthermore, the effects of ChTX or PD98059 treatment on FIK channel activity are not sufficient to drive complete myogenic differentiation, thus supporting a role for FIK in controlling early (ACH receptor channel expression) rather than late (MHC expression) myogenic events. Thus, these data support the idea that bFGF and TGF-β, through activation of the MEK signaling cascade, regulate FIK activity and thereby alter cell growth (in the case of bFGF) and early myogenic events (summarized in Table I).

Since FIK channel activity is necessary for both bFGF- and TGF-β-induced myogenic suppression (Figs. 2 and 3), we hypothesized that the low FIK channel activity in EGFi-treated cells would be insufficient to suppress differentiation in 10T1/2-MRF4 cells. Fig. 3 confirms that EGF failed to suppress ACh channel expression (115.6 ± 31.5 pA/pF, n = 28). Ach channel expression was further increased to 171.6 ± 35.1 pA/pF, n = 32, by ChTX block of the low levels of EGF-induced FIK. In addition to increased ACh channel current densities, 82% of EGFi-treated cells stained positive for MHC and fused into multinucleate myofibers (Fig. 3). These results suggest that the failure of EGF to suppress myogenesis results in part from the absence of elevated FIK channel activity.

**DISCUSSION**

bFGF and TGF-β play critical roles in regulating the onset of myogenic differentiation. The similarities between the myo-
ACh channel expression, we propose that FIK regulates myogenesis independently of its effects on cell proliferation. These data also support the idea that growth factor-mediated FIK expression serves as a general mechanism for suppressing myogenesis.

Our studies identify a critical role for FIK in regulating the fundamental cell biological events of proliferation and differentiation. They provide the first evidence for Ras/MEK/ERK-mediated coupling between receptor tyrosine kinase and TGF-β receptor families and FIK in the physiological control of early myogenic events. Additional evidence for the role of Ras/MEK/ERK in myogenesis has been demonstrated in a study by Bennett and Tonks (17), in which ERK inactivation was found to be necessary for the removal of growth factor-induced suppression of myogenesis in C2C12 cells. It also has been suggested that activation of additional signaling pathways distinct from Ras/MEK/ERK may be necessary for complete myogenic suppression (9). The finding that blocking either Ras/MEK/ERK signaling or FIK channel activity only partially relieves bFGF- or TGF-β-induced myogenic suppression supports the idea that additional mechanisms may exist to regulate later myogenic events. FIK, however, clearly mediates the transition between proliferation and differentiation in 10T1/2-MRF4 cells. FIK also positively regulates proliferation in other cell types (18, 19), suggesting that it may have a corollary inhibitory effect on differentiation in these cells as well as in other tissues in which it is expressed (20–22). The finding that FIK contributes to, and is affected by, the biochemical signaling pathways that regulate cell proliferation and differentiation supports this view.

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FIG. 4. Schematic representation of the role of FIK in bFGF and TGF-β regulation of myogenesis in 10T1/2-MRF4 cells. A, bFGF or TGF-β stimulation up-regulates FIK via the Ras/MEK/ERK signaling pathway and suppresses MRF4-dependent muscle gene expression. MRF4 activation is assayed by measuring the presence or absence of ACh receptor channel expression. B, in the absence of growth factor FIK expression is down-regulated and the MRF4-dependent myogenic program is initiated. C, PD98059 inhibits MEK/ERK signaling, down-regulating FIK and initiating MRF4-dependent transcription. D, ChTX block of FIK channel activity reverses myogenic suppression by bFGF or TGF-β, allowing ACh receptor expression.
Myogenic Inhibition via RAS/MEK/ERK and FIK

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