Regulation of Myogenesis by Fibroblast Growth Factors Requires Beta-Gamma Subunits of Pertussis Toxin-Sensitive G Proteins

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Terminal differentiation of skeletal muscle cells in culture is inhibited by a number of different growth factors whose subsequent intracellular signaling events are poorly understood. In this study, we have investigated the role of heterotrimeric G proteins in mediating fibroblast growth factor (FGF)-dependent signals that regulate myogenic differentiation. Pertussis toxin, which ADP-ribosylates and inactivates susceptible G proteins, promotes terminal differentiation in the presence of FGF-2, suggesting that G\alpha or G\beta\gamma subunits or both are involved in transducing the FGF-dependent signal(s) that inhibits myogenesis. We found that G\beta\gamma subunits are likely to be involved since the expression of the C terminus of beta-adrenergic receptor kinase 1, a G\beta\gamma subunit-sequestering agent, promotes differentiation in the presence of FGF-2, and expression of the free G\beta\gamma dimer can replace FGF-2, rescuing cells from pertussis toxin-induced differentiation. Addition of pertussis toxin also blocked FGF-2-mediated activation of mitogen-activated protein kinases (MAPKs). Ectopic expression of dominant active mutants in the Ras/MAPK pathway rescued cells from pertussis toxin-induced terminal differentiation, suggesting that the G\beta\gamma subunits act upstream of the Ras/MAPK pathway. It is unlikely that the pertussis toxin-sensitive pathway is activated by other, as yet unidentified FGF receptors since PDGF (platelet-derived growth factor)-stimulated MM14 cells expressing a chimeric receptor containing the FGF receptor-1 intracellular domain and the PDGF receptor extracellular domain were sensitive to pertussis toxin. Our data suggest that FGF-mediated signals involved in repression of myogenic differentiation are transduced by a pertussis toxin-sensitive G-protein-coupled mechanism. This signaling pathway requires the action of G\beta\gamma subunits and activation of MAPKs to repress skeletal muscle differentiation.

Of the soluble growth factors thought to play critical roles in the development of skeletal muscle, fibroblast growth factors (FGFs), Sonic hedgehog, scatter factor/hepatocyte growth factor, and transforming growth factor \beta are thought to be required for skeletal muscle development in vivo (2, 5, 16, 23, 48). We are attempting to delineate the signaling pathways utilized by FGFs that regulate the proliferation and differentiation of skeletal muscle cells. Previous studies performed by other groups as well as data obtained in our laboratory have demonstrated that (i) distinct FGF pathways are involved in regulating MM14 myoblast growth and differentiation (37), (ii) FGF signaling pathways cannot be replaced by stimulation of other growth factor receptors (36, 38, 39), and (iii) FGFs stimulate activation of mitogen-activated protein kinase (MAPK) pathways (8, 36, 45, 47). Of the four identified FGF receptor tyrosine kinases, only one, FGF receptor-1, is detectably expressed in MM14 cells (37, 63); it is required for FGF-mediated repression of terminal differentiation (22). Additionally, high-affinity binding and subsequent signaling events require that FGFs bind to both the tyrosine kinase and a heparan sulfate proteoglycan (49, 53, 54).

Pertussis toxin-sensitive, G-protein-coupled mechanisms have been reported to affect myoblast differentiation and proliferation, although the mechanisms involved have not been investigated (30, 67). Pertussis toxin (PT), a protein virulence factor produced by Bordetella pertussis, is composed of an A protomer and a B oligomer. The A protomer consists of a single peptide that ADP-ribosylates specific eucaryotic G proteins (G\alpha\omega), locking the G protein in the GDP-bound state and preventing dissociation of G\alpha and G\beta\gamma subunits, thus leading to inactivation of the G-protein signal. The B oligomer binds to cell surface receptor proteoglycans and transfers the A protomer to the interior of the cell (29).

The heterotrimeric G proteins are composed of distinct \alpha, \beta, and \gamma subunits, and all three can participate in signal transduction. Following receptor activation by agonist, G\alpha subunits of PT-sensitive proteins transmit signals to adenylyl cyclase and other effector molecules (66). The G\beta\gamma heterodimer, released upon activation of PT-sensitive G proteins, activates K+ channels (35), mediates the translocation of the \beta-adrenergic receptor kinase 1 (\betaARK1) (64), regulates specific isoforms of adenylyl cyclase (62) and phospholipase C (PLC) (9), and stimulates the MAPKs (12, 15, 41). Stimulation of MAPK activity by the insulin-like growth factor 1 (IGF-1) receptor tyrosine kinase depends on participation of G\beta\gamma subunits derived from PT-sensitive G proteins (41). As for the G-protein-coupled receptor-mediated pathways, IGF-1 signaling can be inhibited by PT treatment or by a G\beta\gamma subunit inhibitor (41).

A large number of polypeptide growth factor receptors stimulate activation of MAPKs (4, 46, 55). A few reports have demonstrated that MAPK stimulation is PT sensitive. Among the receptor tyrosine kinases, PT interferes with the activation of MAPK by epidermal growth factor in hepatocytes (20) and by IGF-1 in Raf-1 fibroblasts (66). Activation of MAPKs is known to occur via the Ras/Raf/MKK1/2 pathway (27, 60). Recently, MAPKs were reported to be activated by Ras-independent mechanisms that include c-Src protein tyrosine kinase (18) and protein kinase C (7, 44, 46, 65) pathways. Additional complexity in these signaling pathways is suggested by the existence of MAPK kinase kinases other than Raf (3). G-protein-dependent signaling can be coupled to the MAPK
cascade through release of free βγ subunits, which is linked to activation of a Ras-dependent pathway (32), or through activation of MAPK by PT-sensitive Gα subunits (31, 64).

Here we report that PT stimulates myogenic differentiation in the presence of FGF-2, inhibits FGF-induced proliferation of MM14 cells, and blocks FGF-2-stimulated MAPK activity. In addition, FGF-2 signaling can be blocked by inhibitors of Gβγ subunits. Expression of the free Gβγ dimer suppresses PT-stimulated differentiation and mimics the effect of FGF-2 on MM14 cells. Thus, we demonstrate for the first time that signaling pathways regulated by binding of FGF-2 to FGF receptor-1 can be mediated by Gβγ subunits of PT-sensitive heterotrimeric G proteins.

MATERIALS AND METHODS

Cell culture. Mouse MM14 cells (39) were cultured on gelatin-coated plates in growth medium consisting of Ham’s F10 (Life Technologies, Gaithersburg, Md.) supplemented with 0.8 mM CaCl2, 100 U of penicillin G per mL, 5 μg of streptomycin sulfate per mL, and 15% horse serum. The concentration of FGF-2 was increased from 0.3 to 2.5 nM with increasing cell density. Human recombinant FGF-2 was purified from a yeast strain expressing this growth factor (33). PT and cholera toxin (CT) were purchased from Life Technologies. B oligomer of PT was purchased from Calbiochem (San Diego, Calif.), and forskolin was purchased from Sigma (St. Louis, Mo.).

Cloning and growth medium. Cells were plated onto six-well plates at a density of 50 cells per well in growth medium containing 0.3 nM FGF-2, cultured for 48 h, then fixed with 70% ethanol and 37% formaldehyde-glacial acetic acid, 20:1 at 4°C, and immunostained for myosin heavy chain (MHC) as previously described (56). Cells were analyzed by phase-contrast microscopy. The number of nuclei per colony was determined, and percent MHC-positive cells per well was quantified.

DNA transfection. DNA was transiently transfected into MM14 cells by a calcium phosphate-DNA precipitate method as described previously (36). The expression vector pBS5 PDGFR/FGFR1, encoding a chimeric platelet-derived growth factor (PDGF) β receptor/FGFR1 construct (PDGF/FGF receptor chimera), is composed of the PDGF β-receptor extracellular domain and the FGF receptor-1 transmembrane and intracellular domains. This vector was previously constructed in our laboratory (37). Eukaryotic expression vectors pCDM8.1G1 and pCDM8.1G2, encoding G1 (17) and G2 (19), respectively, were a gift from M. Simon (California Institute of Technology). pCEV CD8 βARK, an expression vector that encodes a membrane-targeted C-terminal fragment of BARK1 (11), and a control vector (pCEV CD8) were provided by S. Gururangan (National Institute of Dental Research, National Institutes of Health). MMTV-LTR Ras E66, carrying the Ha-ras oncogene (51), RSV-Raf-BXB, carrying a constitutively active form of the ras-1 proto-oncogene, Raf-BXB (6) (referred to as BXB-Raf in this report), and CMV-MKK1(R4F), a cytoskeletal virus (36) (referred to as recombinant BXB-Raf), were provided by R. Palmer (Howard Hughes Medical Institute, University of Washington), U. Rapp (National Cancer Institute, Frederick Cancer Research and Development Center), and N. Ahn (Howard Hughes Medical Institute, University of Washington). BXB-Raf contains a temperature-sensitive mutation in the MAPK kinase 1 (RAF-MKK1) (43), were provided by R. Palmer (Howard Hughes Medical Institute, University of Washington), U. Rapp (National Cancer Institute, Frederick Cancer Research and Development Center), and N. Ahn (Howard Hughes Medical Institute, University of Washington).

Muscle-specific promoter assay. A differentiation-sensitive muscle-specific reporter activity assay was used to determine the extent of MM14 differentiation following transient transfection. The reporter contained the firefly luciferase gene driven by a muscle-specific promoter (MSP, human α-cardiac actin promoter) (36). MM14 cells were plated on six-well plates at a density of 10,000 cells/well and cotransfected with 1 μg of MSP reporter vector, 1 μg of CMV-LacZ, and different amounts of expression vector or control vector as indicated. Equivalent DNA concentrations were maintained by the addition of pcDNA3 vector (Invitrogen, San Diego, Calif.). Cells were harvested and luciferase activity was determined 36 h following transfection. Luciferase activity was determined by using a Tropix (Bedford, Mass.) Dual Light assay kit and quantitated following FGF stimulation or treatment. Luciferase activity was determined, quantitated, and normalized as described for the luciferase assay.

RESULTS

Proper development and regeneration of skeletal muscle in vivo is likely to be dependent on FGFs (16, 24). MM14, a skeletal muscle satellite cell line, like skeletal muscle primary cultures, is dependent on FGFs (10, 52, 59). MM14 cells thus serve as a model for investigating signaling in primary cells. We have previously demonstrated that ERK1/2 (extracellular-regulated kinases 1 and 2) can be activated by FGF-2 in MM14 cells (36). We wanted to investigate further the signaling mechanisms activated by FGF in MM14 cells and to identify pathways involved in the regulation of myogenesis, specifically G-protein signaling. We therefore treated MM14 cells with PT, CT, and forskolin to examine whether cyclic AMP-dependent signaling plays a role in the FGF response. While PT blocked FGF activity and promoted terminal differentiation in a dose-dependent fashion (Fig. 1), neither CT, which ADP-ribosylates G proteins involved in adenylyl cyclase activation, nor forskolin, a direct activator of adenylate cyclase (25), affected the proliferation or differentiation of MM14 cells (Fig. 1A). These data suggest that the action of PT is distinct from its potential effects on adenylyl cyclase and protein kinase A. The B oligomer of PT is known to bind membrane proteoglycans (29). To rule out a possible effect of the B oligomer on FGF binding to its receptor complex, we examined whether the B oligomer of the holotoxin was sufficient to induce skeletal muscle differentiation. Treatment of MM14 cells with the B oligomer over a wide range of concentrations elicited no detectable effect on MM14 cell differentiation (Fig. 1B). In contrast to the B oligomer, treatment with the PT holotoxin promoted myogenesis under identical culture conditions, demonstrating that the effect of the toxin is likely to be mediated via the ADP-ribosylation of a Gα protein(s) (Fig. 1B). Consistent with the ability of PT to block FGF signaling events that repress myogenesis, PT treatment also prevented proliferation in the presence of FGF-2 and 15% horse serum (Fig. 2). Neither forskolin, CT, nor the B oligomer of PT had any detectable effect on MM14 cell proliferation (Fig. 2).

To determine whether PT directly interfered with signaling from FGF receptor-1, we studied the PT sensitivity of MM14 cells transiently transfected with a construct encoding a PDGF receptor chimera (37). MM14 cells do not express endogenous PDGF receptors (36), and expression of the chimeric receptor confers PDGF-BB-dependent inhibition of myogenic differentiation in MM14 cells (37). In the presence of PDGF-BB, PT induces differentiation of MM14 cells transiently transfected with the chimeric receptor (Fig. 3). These data suggest that PT inhibits signals transduced directly from activation of the FGF receptor-1 tyrosine kinase.

Recent data have shown that G-protein-coupled mechanisms of signal transduction often require the Gβγ subunits (14). Expression of a specific Gβγ subunit binding peptide derived from the carboxyl terminus of βARK1 (βARK1-CT) can block Gβγ subunit-mediated signal transduction in stably and transiently transfected cell lines (11, 33). The βARK1-CT fragment is localized to the cell membrane by the fusion of βARK1-CT to the transmembrane domain from the CD8 receptor (βARK-CDS), thus effectively excluding Gβγ subunits from participating in intracellular signaling. To determine whether the inhibition of myogenic differentiation was mediated by Gβγ or Go subunits, we examined the effects of transient expression of βARK-CT on MM14 cell differentiation.

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Transient expression of this Gβγ-sequestering agent stimulated differentiation in the presence of added FGF-2, as assayed by induction of a muscle-specific promoter (Fig. 4). Transfection with a control vector containing the coding sequences for the CD8 transmembrane domain but lacking the βARK-CT sequences elicited no detectable effect, indicating that the induction of differentiation was likely to be due to Gβγ subunit sequestration (Fig. 4).

If Gβγ subunits are critical for transducing FGF signals in skeletal muscle cells, then expression of the appropriate Gβγ subunits would be expected to substitute for FGF. Transfection of MM14 cells with increasing amounts of either a Gβγ subunit expression vector inhibited terminal differentiation, similar to what was observed for control cells given FGF-2 (Fig. 5A). A similar experiment was performed in the presence of FGF-2 and PT. As expected, transient transfection of Gβγ subunits rescued MM14 cells from PT-stimulated differentiation (Fig. 5B). However, transfection with Gy2 alone elicited no detectable effect, while transfection with only Gβ1 consistently increased MSP activity to levels greater than those for cells treated with PT alone (Fig. 5B). Taken together, these data suggest that the Gβγ subunits play a central role in FGF-dependent regulation of myogenesis.

As a further measure of the dependence of FGF signaling on a Gβγ-dependent mechanism, we examined the ability of FGF to activate MAPKs in cells pretreated with PT. The PathDetect Elk system detects MAPK activation by phosphorylation of an Elk transcriptional activator fragment (amino acids 307 to 428) fused to the GAL4 DNA binding domain (68). Phosphorylation of this fusion protein by MAPKs then activates a reporter pFR-Luc consisting of the firefly luciferase gene placed downstream of a basic promoter element and located 3' to five tandem repeats of the 17-bp GAL4 binding element. Control experiments with cells transiently cotransfected with either pFA-EIk1 or pFR-Luc alone, or with the combination of pFR-Luc with the transactivating vector lacking the EIk1 domain, displayed no luciferase activity (data not shown). MM14 cells transiently cotransfected with pFR-Luc and pFA-EIk1 were stimulated with FGF-2. Upon FGF-2 stimulation, a 4.0- to 7.5-fold increase in MAPK activity was observed, consistent with our previous observations (36). Pretreatment of MM14 cells with PT completely abolished MAPK activation, while pretreatment with forskolin and CT had minimal effects on MAPK activity (Fig. 6). The effects of PT treatment on MAPK activity and differentiation suggest that a Gβγ protein-dependent pathway may be involved in activation of MAPKs following FGF stimulation. To test this hypothesis, we examined whether the PT-
induced block in FGF signaling could be overcome by known activators of the Ras/MAPK pathway. Activators of the MAPK pathway including Ha-Ras (Ej6-Ras), Raf (BXB-Raf), and MKK1 (R4F-MKK1) all activate the Elk1 reporter system in MM14 cells in the presence of PT (Fig. 7A). Moreover, these MAPK pathway activators repress differentiation in the presence or absence of FGF (Fig. 7B), suggesting that they act on signaling pathways directly involved in regulating terminal differentiation. The observation that constitutively active mutants of Ras (Ej6-Ras), Raf (BXB-Raf), and MKK1 (R4F-MKK1) all overcome PT-induced differentiation as well as MAPK activity suggests that the G_i/o proteins inhibited by PT act in a pathway parallel to a MAPK cascade or more likely at an early step in an FGF signaling cascade.

DISCUSSION

The molecular mechanisms involved in the regulation of skeletal muscle differentiation by members of the FGF family are poorly understood. We and others have previously demonstrated that skeletal muscle cells, which are dependent on FGFs, stimulate ERK1/2 activity (1, 8, 36, 45, 47). To better understand the events leading to activation of MAPKs, we have examined the role of G_i/o proteins in FGF receptor-1 signaling. In this study, we found that PT induced differentiation, clearly blocking the effects of FGF-2. The induction of differentiation was specific and dose dependent. Neither CT, which ADP-ribosylates PT-insensitive G proteins involved in adenylate cyclase activation, nor forskolin, a direct activator of adenylate cyclase (25), affected the growth or differentiation of MM14 myoblasts. The biological activity of PT is usually due to the S1 subunit, which ADP-ribosylates G_i/o proteins (29). However, the binding of the B oligomer to cell surface proteoglycans can increase inositol triphosphate production and intracellular calcium levels in Jurkat cells (57), stimulate proliferation in human T lymphocytes (21), and enhance glucose oxidation in adipocytes (61). The B oligomer does not detectably affect myogenic differentiation or proliferation in MM14 skeletal muscle myoblasts, demonstrating that the effect of PT is likely to be mediated by the activity of the S1 subunit, which ADP-ribosylates G_i/o protein(s). Thus, in MM14 cells, as in other cells (40, 56, 58), FGF-dependent signals appear to require the action of a PT-sensitive G_i/o protein(s). We have previously demonstrated that FGF-dependent repression of differentiation in MM14...
cells requires a functional FGF receptor-1 (22), the only detectable FGF receptor isoform expressed in MM14 cells (37, 63). The capacity of a truncated dominant negative FGF receptor-1 mutant to block FGF signaling and promote differentiation in these cells demonstrates that repression of myogenic differentiation by FGF requires FGF receptor-1 (22). Furthermore, the PDGF/FGF receptor chimera is capable of repressing differentiation in the absence of FGFs and in the presence of a dominant negative FGF receptor mutant (37). With few exceptions, PT-sensitive Gβγ-mediated signal transduction events are usually initiated by binding of a specific ligand to a membrane-spanning G-protein-coupled receptor. In this report, we demonstrated that repression of myogenic differentiation upon addition of PDGF-BB to cells expressing the PDGF/FGF receptor chimera is PT sensitive. Thus, the FGF receptor-1 tyrosine kinase appears to mediate signals via a PT-sensitive Gαi/o protein(s). Furthermore, it is unlikely that an FGF receptor other than FGF receptor-1 is involved.

Upon ligand-dependent receptor activation and binding of GTTP to the α subunit of G proteins, Go and Gβγ subunits dissociate. In this active state, both α and βγ subunits can activate or inhibit their effectors and thus participate in intracellular signaling. We demonstrated that expression of a specific Gβγ subunit binding peptide derived from BARK1-CT induced myogenic differentiation in the presence of FGF-2. Moreover, transient transfection of Gβγ subunits rescued MM14 cells from PT-stimulated differentiation and prevented differentiation in the absence of added FGF-2. Expression of Gβ1 or Gγ2 inhibited MSP activity at the highest concentrations tested; alone, each was capable of reducing MSP activity by only 25%. However, coexpression of both subunits elicited a synergistic effect and reduced MSP activity by 78% (~3-fold) in the absence of added FGF-2. These data suggest that the levels of Gβγ subunits involved in FGF signaling may be limiting since neither subunit alone was effective at reducing MSP activity.

In the presence of PT, overexpression of Gγ2 had no effect but overexpression of Gβ1 enhanced MSP activity 1.8-fold. In contrast to the effects of either subunit transfected individually, overexpression of both Gβ1 and Gγ2 rescued the PT-induced block of FGF signaling and reduced MSP activity to control levels in the absence of PT. We propose that specific combinations of Gβγ subunits may be required for FGF signaling in skeletal muscle myoblasts. Thus, overexpression of an individual Gβ or Gγ subunit could negatively or positively affect FGF signaling, depending on the concentration and distribution of Gβ and Gγ subunits within the cell.

The molecular mechanisms involved in activation of Gβγ by FGF are unclear, as are the downstream targets of Gβγ in skeletal muscle cells. In other cell types, the participation of Gβγ signaling in tyrosine kinase-mediated activation of ERKs requires calcium and/or PLCs (13). Preliminary data from our laboratory also suggest that PLCs are required for FGF signaling and that PLC activation follows stimulation of Gβγ by FGFs (unpublished data). A potential mechanism for coupling Gβγ with the Ras/MAPK cascade in skeletal muscle cells may...
also involve regulation of Ras. The pleckstrin homology (PH) domain shared by several proteins that regulate the activity of p21ras, including Ras–GDP-releasing factor, Ras–GTPase-activating protein, and IRS-1, binds Gβγ subunits (64, 69). Interactions between Gβγ subunits and the PH domains of one or more p21ras-regulatory proteins may provide the coupling of Gβγ subunit-mediated signaling to activation of MAPKs, thereby inhibiting myogenic differentiation. Recently, FRS2, a potential substrate for FGF receptor-1, was identified in fibroblasts (34). It is not yet known if FRS2 is present in skeletal muscle cells or if phosphorylation of FRS2 is dependent on G-protein activation. However, it is interesting that Gβγ subunits bind to a similar substrate for the insulin receptor through the PH domains (64). Alternatively, Gβγ subunits may directly or indirectly affect Ca2+ channels and activate Ras-and/or MAPK-dependent pathways through modulation of intracellular Ca2+ (42, 50). Recently, a second mechanism involving Ras-independent stimulation of MAPKs via Goαs was described (66).

Activation of the MAPK cascade(s) is widely considered to be essential for growth factor-induced proliferation responses. To obtain further data in support of PT-sensitive G-protein involvement in MAPK activation, we examined induction of an Elk1-dependent reporter gene in MM14 cells cultured without the growth factor (A) or to activity in cells cultured in the presence of 0.3 nM FGF-2 (B) is shown. Mean values and standard deviations represent three (A) and two (B) independent experiments performed in triplicate.

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