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Role of insulin-like growth factors and myogenin in the altered program of proliferation and differentiation in the NFB4 mutant muscle cell line

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ABSTRACT **In the present study we used the mutant muscle cell line NFB4 to study the balance between proliferation and myogenic differentiation. We show that removal of serum, which induced the parental C2C12 cells to withdraw from the cell cycle and differentiate, had little effect on NFB4 cells. Gene products characteristic of the proliferative state, such as c-Jun, continued to accumulate in the mutant cells in low serum, whereas those involved in differentiation, like myogenin, insulin-like growth factor II (IGF-II), and IGF-binding protein 5 (IGFBP-5) were undetectable. Moreover, NFB4 cells displayed a unique pattern of tyrosine phosphorylated proteins, especially in low serum, suggesting that the signal transduction pathway(s) that controls differentiation is not properly regulated in these cells. Treatment of NFB4 cells with exogenous IGF-I or IGF-II at concentrations shown to promote myogenic differentiation in wild-type cells resulted in activation of myogenin but not MyoD gene expression, secretion of IGFBP-5, changes in tyrosine phosphorylation, and enhanced myogenic differentiation. Similarly, transfection of myogenin expression constructs also enhanced differentiation and resulted in activation of IGF-II expression, showing that myogenin and IGF-II cross-activate each other's expression. However, in both cases, the expression of *Jun* mRNA remained elevated, suggesting that IGFs and myogenin cannot overcome all aspects of the block to differentiation in NFB4 cells.**

Differentiation of myoblasts to form postmitotic myotubes has traditionally been viewed as being negatively controlled by mitogens (1). Differentiation is inhibited in myoblasts cultured in high concentrations of serum or in the presence of the serum components basic fibroblast growth factor or transforming growth factor β. By default, removal of serum leads to withdrawal from the cell cycle, activation of the expression of muscle-specific structural genes, and cell fusion to form multinucleated myotubes. However, more recently it has been shown that the family of peptides that are structurally related to insulin, the insulin-like growth factors (IGFs), plays an active role in controlling myogenic differentiation (for review see refs. 2 and 3). IGFs are growth factors for myoblasts, as well as potent stimulators of myogenic differentiation, and may even be essential for the latter process to occur (4–6). IGF-I appeared to be a more potent stimulator of myoblast proliferation than IGF-II, as differentiation of myoblasts following IGF-I treatment lagged behind those treated with IGF-II (7). Several groups have demonstrated that IGF expression, particularly IGF-II, increased during myoblast differentiation in response to serum withdrawal (8–11) and that the amount of IGF-II secreted correlated with the rate of spontaneous differentiation (6). Addition of basic fibroblast growth factor to low-serum medium inhibited IGF-II expression and blocked differentiation (12). Similarly, antisense oligonucleotides complementary to IGF-II mRNA inhibited differentiation in the absence but not in the presence of exogenous IGF-II (6). Thus, it has been proposed that IGFs act in an autocrine and/or paracrine manner on myoblasts to promote myogenic differentiation.

Myogenic differentiation promoted by IGFs has been shown to require induction of the myogenic regulatory factor myogenin (6, 13). This transcription factor, a member of the helix–loop–helix family of myogenic regulators which also includes MyoD, myf-5, and MRF4, binds to enhancers of muscle-specific genes and activates their expression (for review see ref. 14). Antisense oligonucleotides to myogenin mRNA blocked IGF-I-mediated stimulation of myogenic differentiation but had no effect on stimulation of proliferation, suggesting that IGF-I activates multiple signal transduction pathways in myoblasts and that myogenin is an essential intermediate only in the differentiation pathway (15). Paradoxically, Brown *et al.* (16) and Rosen *et al.* (11) have shown that the increase in IGF-II mRNA levels upon withdrawal of serum occurred after activation of the myogenin gene. Therefore, the relationship between IGF-II and myogenin gene expression during myoblast differentiation is unclear.

In muscle, IGF-I and IGF-II interact primarily with the IGF-I receptor, a transmembrane tyrosine kinase (17). The IGFs also interact with secreted and cell surface associated IGF-binding proteins (IGFBPs) that can enhance or inhibit IGF activity in cultured cells (for review see refs. 18 and 19). IGFBP-4, -5, and -6 are the most abundant binding proteins expressed by muscle cells *in vitro*, and differentiation of myoblasts into myotubes is accompanied by a dramatic induction in IGFBP-5 production (20, 21). Given that IGF-I and -II have multiple effects on muscle, it is likely that the complexity of responses of muscle cells to IGFs is generated by interaction with this array of potential receptors and binding proteins.

We previously characterized a nondifferentiating muscle cell line, NFB, that appears quite useful in studying the role of IGFs in controlling myogenic differentiation and in dissecting the signal transduction pathways that control proliferation and differentiation of muscle cells. Heterokaryon analysis showed that the mutant phenotype was dominant: fusion of NFB cells with normal muscle cells inhibited myogenesis in the normal cells (22). We recently isolated a nondifferentiating subclone of NFB cells, NFB4, in which accumulation of MyoD, myogenin, and IGF-II mRNAs is undetectable. Moreover, whereas withdrawal of serum from wild-type cells resulted in changes in the constellation of tyrosine-phosphorylated proteins, no such changes occurred in NFB4 cells. Addition of IGFs or transfection of cells with MyoD or myogenin expression constructs significantly enhanced differentiation in NFB4 cells and showed that myogenin and IGF-II cross-activate each other's expression. Although these treatments resulted in a

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Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein.

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partial rescue of the mutant phenotype, the expression of the *Jun* gene remained elevated, suggesting that conflicting signal transduction pathways are active in NFB4 cells under these conditions.

MATERIALS AND METHODS

Cell Culture and Transfections. NFB4 is a subclone of the nondifferentiating NFB cell line originally derived from the C2C12 mouse muscle cell line by treatment with ethyl methansulfonate (22). Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (vol/vol) fetal bovine serum and 5% (vol/vol) defined bovine serum (HyClone) (GM) at 37°C in a humidified 10% CO_2/90% air atmosphere. To induce differentiation, cells were washed with serum-free DMEM and maintained in DMEM containing 2% (vol/vol) horse serum (HyClone) (DM) for 3 days. For IGF treatment, several concentrations of human IGF-I (Boehringer Mannheim) and IGF-II (Mallinckrodt) were tested, and those found to be most effective in promoting differentiation were 15 ng of IGF-I and 100 ng of IGF-II per ml in DMEM with 0.1% bovine serum albumin in serum-free media, consistent with the work of Florini *et al.* (23). Medium was changed every day, and cells were harvested on the third day as described below.

Introduction of expression constructs into NFB cells was performed by the calcium phosphate coprecipitation method, as described (22). Approximately 10^6 cells were cotransfected with 15 μg of a plasmid containing the mouse MyoD or myogenin cDNAs under the control of the mouse sarcoma virus long terminal repeat, together with 1.5 μg of pSV2neo. The DNA was removed 24 h later with the addition of fresh growth medium. After an additional 24 h, cells were split 1:4 and refed with medium to which 400 μg of G418 (Geneticin; GIBCO/BRL) per ml had been added. Pooled G418-resistant clones were picked 14 days later and assayed for their ability to differentiate in DM.

RNA Isolation and Northern Analysis. Total cellular RNA was isolated essentially as described by Chomczynski and Sacchi (24). Northern blots were performed as described (22). The following cDNAs were labeled by the random primer method by using a Bio-Rad kit to generate probes: a 1.1-kb *Eco*RI restriction fragment from mouse myogenin (25), a 1.9-kb *Eco*RI fragment from mouse MyoD (26), a 3.2-kb *Bam*HI–*Hin*dIII Myc fragment from pM104BH (27), a 0.8-kb *Hpa*I–*Bam*HI fragment from human JUN and a 1.2-kb *Bam*HI–*Kpn* I fragment from human FOS (28), a 0.3-kb fragment between *Pvu* II (at Ser-50 of the coding sequence) and *Bal* I of human IGF-II (29), and a 0.78-kb *Eco*RI human IGF-I fragment, originally from Casella *et al.* (30).

Immunohistochemistry. Cells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl/2.7 mM KCl/4.3 mM $Na_2HPO_4 \cdot 7\ H_2O$/1.4 mM KH_2PO_4), fixed with 1% formaldehyde in PBS for 1 h at room temperature, and then treated with ice-cold methanol for 20 min. Muscle myosin heavy chain was detected by using 4A.1025 antibody (31). Cells were incubated with undiluted hybridoma tissue culture supernatant for 1 h at room temperature followed by incubations with biotinylated anti-mouse IgG (H + L) (Vector Laboratories) diluted 1:400 in PBS/0.1% Tween 20, and avidin–biotin complex horseradish peroxidase (Vector Laboratories ABC kit). Horseradish peroxidase reactivity was visualized by using 1 mg of diaminobenzidine per ml of 50 mM Tris·HCl, pH 7.2/0.03% H_2O_2/0.03% $CoCl_2$.

Western Analysis. After incubation and stimulation of cells, 10-cm culture dishes of confluent cells were placed on ice, washed twice with PBS, and lysed in 0.5 ml of cold lysis buffer (50 mM Hepes, pH 7.4/150 mM NaCl/1.5 mM $MgCl_2$/1 mM EGTA/100 mM NaF/10 mM sodium pyrophosphate/1 mM phenylmethylsulfonyl fluoride/2 mg each of leupeptin and aprotinin per ml/1 mM Na_3VO_4/1% Triton X-100) for 10 min with shaking. All manipulations of cell lysates were at 4°C. Lysates were scraped into Eppendorf tubes and cleared of nuclei and detergent-insoluble material by centrifugation for 10 min at 12,400 × *g*. Protein concentration was measured by using the BCA kit (Pierce). The samples (60 μg of protein) were separated by electrophoresis through SDS/7.5% polyacrylamide gels and electrophoretically transferred to Immobilon-P membranes (Millipore). Blots were blocked for 90 min in 5% (wt/vol) bovine serum albumin in PBS/0.5% Tween 20 (PBST). Anti-phosphotyrosine monoclonal antibody 6G9 (GIBCO/BRL), diluted 1:2000 in PBST, was applied for 1 h at room temperature. Blots were washed five or six times with PBST for 40 min before being incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Pierce), diluted 1:2000 in PBST with 3% (wt/vol) nonfat dry milk. After blots were washed as described above, phosphotyrosine proteins were detected with the Renaissance Chemiluminescence Reagent (DuPont/NEN).

Ligand Western analysis was performed as described (32). Cells in GM were seeded at 10^5 cells per ml; cells in DM or with IGF-I were seeded at 10^6 cells per ml. After 3 days, samples of conditioned medium (2 μl) were separated by SDS/12% polyacrylamide gel electrophoresis under nonreducing conditions, transferred to nitrocellulose, and probed with ^{125}I-labeled IGF-I. IGF-I was iodinated by using Iodo-Beads (Pierce) according to the manufacturer's recommendations.

RESULTS

NFB4 Cells Demonstrated Altered Accumulation of Gene Products Involved in Controlling Proliferation and Differentiation. We utilized the NFB4 mutant muscle cell line to delineate the relative roles of IGFs and the myogenic helix–loop–helix transcription factors MyoD and myogenin in controlling differentiation. Northern blot analysis was performed with total RNA isolated from mutant NFB4 and wild-type C2C12 cells maintained in either high-serum-containing GM or low-serum-containing DM (Fig. 1). Transcripts encoding Jun, as well as Myc and Fos (data not shown), were present at comparable levels in the two cell types in GM. However, whereas the protooncogene mRNAs decreased in C2C12 cells in DM, these transcripts remained elevated in NFB4 cells maintained under the same conditions. Consequently, NFB4 cells continued to grow in low-serum, conditions under which wild-type cells withdrew from the cell cycle and differentiated (Fig. 2). Furthermore, no accumulation of IGF-II, MyoD, or

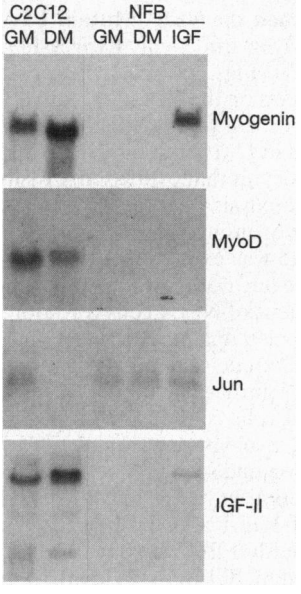

FIG. 1. Northern analysis of cells in GM, DM, or following IGF-I treatment. Total RNA (5 μg) from C2C12 and NFB4 cells cultured under different conditions was analyzed by a Northern blot with the indicated probes under stringent conditions. The filter was stripped between each hybridization. The C2C12 and NFB cells in GM were confluent at the time of RNA isolation. Cells were maintained in DM or treated with IGF-I for 3 days prior to RNA isolation.

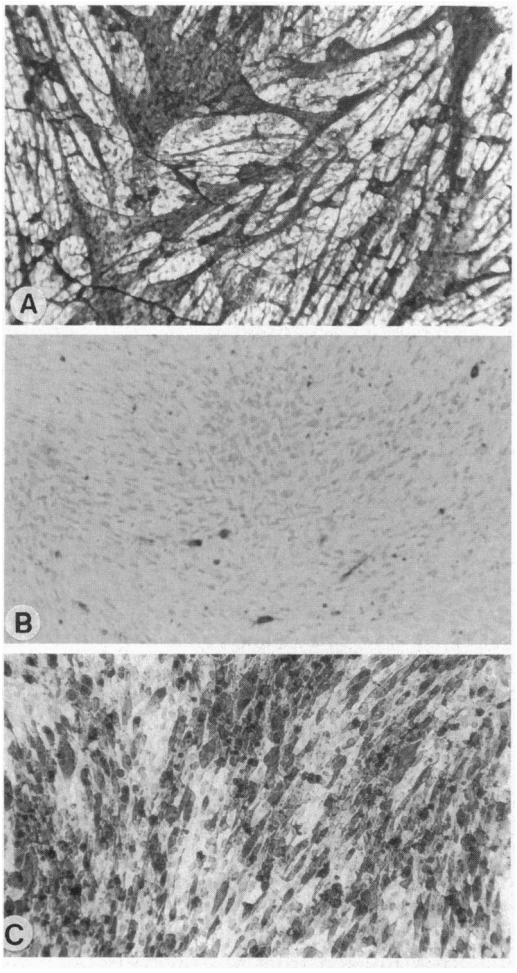

FIG. 2. Immunocytochemical analysis of myosin heavy chain expression in cells in DM or following IGF-I treatment. Upon reaching confluence, C2C12 (*A*) or NFB4 cells (*B*) were maintained in DM for 3 days. NFB4 cells were also treated with IGF-I in serum-free medium for 3 days (*C*). The extent of differentiation was assessed by reactivity with a myosin heavy chain monoclonal antibody.

myogenin mRNAs was detected in NFB4 cells in GM or DM (Fig. 1). IGF-I mRNA was undetectable in C2C12 and NFB4 cells regardless of growth conditions (data not shown). Thus, NFB4 cells maintain a proliferative myoblast phenotype even in the absence of mitogens.

Exogenous IGFs Partially Rescued the NFB4 Mutant Phenotype Through Activation of Myogenin Gene Expression. Treatment of NFB4 cells with IGF-I (Figs. 1 and 2*C*) or IGF-II (data not shown) had dramatic effects on the NFB4 phenotype. Although the cells did not fuse to form multinucleated myotubes as did wild-type C2C12 cells in DM (Fig. 2*A*), ≈50% of the cells formed differentiated myocytes that expressed myosin heavy chain (Fig. 2*C*). Northern analysis of total RNA from IGF-I-treated cells showed that myogenin gene expression was activated and that the IGF-II gene was expressed, albeit to a much lesser extent (Fig. 1). However, no MyoD mRNA was detectable following IGF-I treatment of NFB4 cells. Protooncogene mRNA accumulation was also unaffected, which may in part account for the incomplete rescue of the differentiated phenotype by IGFs. High serum significantly inhibited IGF-I-induced differentiation of NFB4 cells (data not shown).

Because differentiation of C2C12 myoblasts is accompanied by a dramatic elevation in the expression and secretion of IGFBP-5 (20, 21), we analyzed IGFBP secretion in NFB4 cells by ligand Western blotting with ^{125}I-labeled IGF-I as a probe (Fig. 3). Analysis of GM and DM showed that IGFBP-3 (44 and 39 kDa), IGFBP-2 (34 kDa), and to a lesser extent IGFBP-4 (24 kDa), were

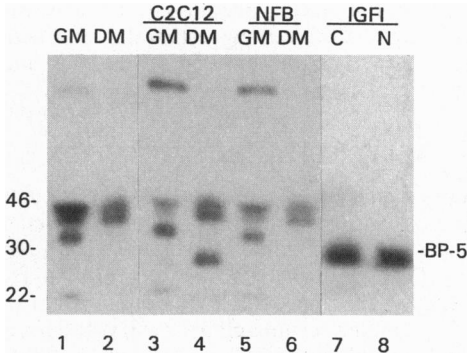

FIG. 3. Ligand Western blot of IGFBPs secreted by C2C12 and NFB cells cultured under different conditions. Proteins were separated by SDS/PAGE under nonreducing conditions, transferred to nitrocellulose, and probed with ^{125}I-labeled IGF-I. Lane 1, nonconditioned GM; lane 2, nonconditioned DM. These lanes served as a control for IGFBPs present in serum. Lane 3, GM conditioned by C2C12 cells; lane 4, DM conditioned by C2C12 cells; lane 5, GM conditioned by NFB4 cells; lane 6, DM conditioned by NFB4 cells; lane 7, serum-free medium supplemented with IGF-I and conditioned by C2C12 cells; and lane 8, serum-free medium supplemented with IGF-I and conditioned by NFB cells. The positions of molecular mass markers (in kDa) are indicated on the left. The position of IGFBP-5 (BP-5) is indicated on the right.

present in serum in the absence of cells (Fig. 3, lanes 1 and 2). Ligand Western analysis of GM or DM conditioned by C2C12 cells showed that secretion of IGFBP-5 (31 kDa) consistently increased during C2C12 differentiation (Fig. 3, lanes 3 and 4). By contrast, IGFBP-5 was not detectable in DM conditioned by NFB4 cells (Fig. 3, lane 6). Interestingly, a large (>80 kDa) IGFBP of unknown identity that was accumulated by both C2C12 and NFB cells in GM was undetected following exposure to DM. Treatment of NFB4 cells with IGF-I resulted in secretion of IGFBP-5 to levels on par with C2C12 cells (Fig. 3, lanes 7 and 8). IGF-I enhanced IGFBP-5 secretion in both cell types. Treated C2C12 and NFB4 cells differed in that a 24-kDa IGFBP, likely to be IGFBP-4 (20), was also secreted, albeit at low levels, by C2C12 cells. Therefore, treatment of NFB4 cells with IGF-I restores IGFBP-5 production to wild-type levels.

Exogenous Myogenin Also Partially Rescued the NFB4 Mutant Phenotype and Led to Increased IGF-II mRNA Expression. The above results showed that IGF-I treatment of NFB4 cells led to normal accumulation of IGFBP-5, activation of the myogenin gene (but not the MyoD gene), and enhancement of myogenic differentiation. We next wanted to determine if providing myogenin or MyoD directly to NFB4 cells would bypass the requirement for IGFs. We showed previously that transfection of NFB cells with MyoD expression constructs rescued the mutant phenotype (22), analogous to treatment with IGFs. NFB4 cells were cotransfected with either a MyoD or a myogenin expression construct together with pSV2neo, and stable transfectants were selected by resistance to G418. RNA isolated from several hundred pooled G418-resistant clones maintained in DM was analyzed by a Northern blot (Fig. 4). Myogenin mRNA was readily detected in both myogenin and MyoD transfectants, indicating that MyoD cross-activated myogenin expression. On the other hand, MyoD mRNA was not detected in myogenin transfectants, confirming the results obtained with IGF-I treatment that myogenin can induce myogenic differentiation independent of MyoD. Also consistent with the phenotype of IGF-I-treated NFB4 cells, Jun mRNA levels remained elevated in cells transfected with MyoD or myogenin. In spite of Jun overexpression, a significant increase in IGF-II transcript accumulation in both populations of transfected cells was observed. No significant difference was apparent between cells expressing myogenin alone or both myogenin and MyoD in

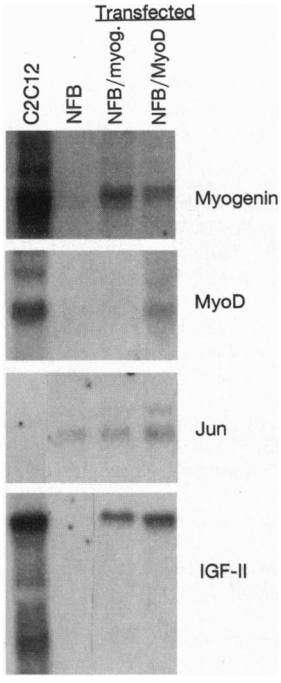

FIG. 4. Northern analysis of myogenin- and MyoD-transfected NFB4 cells. Total RNA (5 μg) from control C2C12 and NFB4 cells and pooled clones of NFB4 cells stably transfected with myogenin or MyoD expression constructs was analyzed by a Northern blot with the indicated probes under stringent conditions. The filter was stripped between each hybridization. All cells were maintained in DM for 3 days prior to RNA isolation.

their ability to differentiate. Taken together, these results indicate that not only does IGF treatment activate myogenin gene expression, but myogenin activates IGF-II expression in NFB4 cells. MyoD also induces IGF-II expression, probably indirectly through cross-activation of myogenin.

NFB4 Mutant Cells Exhibited a Distinct Pattern of Tyrosine-Phosphorylated Proteins That Was Affected by IGF Treatment. One possible explanation of these results is that the signal transduction pathway(s) that controls proliferation and/or differentiation via IGF-II and myogenin expression is not properly regulated in NFB4 cells in response to changing culture conditions. Treatment with IGFs may at least partially circumvent these pathways. To explore this idea, we examined the constellation of tyrosine-phosphorylated proteins present in C2C12 and NFB4 cells cultured under different growth conditions by Western blot analysis with an anti-phosphotyrosine antibody (Fig. 5). Several interesting differences were noted. First, a 97-kDa phosphorylated protein was consistently more abundant in NFB4 cells than in C2C12 cells in GM or DM (Fig. 5, lanes 1–4, 6 and 7). NFB4 cells treated with IGF-I for 24 or 48 h (Fig. 5, lanes 5 and 8) accumulated levels of this protein similar to those in C2C12 cells in DM (Fig. 5, lanes 3 and 6). Second, after 24 h of exposure to DM, the abundance of a phosphorylated protein at 190 kDa increased significantly in C2C12 cells but not in NFB4 cells (Fig. 5, lanes 3 and 4). Treatment of NFB4 cells with IGF-I for 24 h resulted in increased phosphorylation of the 190-kDa protein (Fig. 5, lane 5). Enhanced phosphorylation of the 190-kDa protein was transient in both C2C12 cells in DM and IGF-I-treated NFB4 cells, such that by 48 h, the signal was reduced to levels comparable with NFB4 cells in DM (Fig. 5, lanes 6–8). The identity of the 190-kDa protein is unknown, but immunoprecipitation and Western analysis indicated that it was not insulin receptor substrate 1 or rhoGAP p190 (data not shown). Finally, NFB4 cells maintained in DM for 48 h showed increased phosphorylation of a 38-kDa protein relative to that in either wild-type C2C12 cells in DM or IGF-I-treated NFB4 cells (Fig. 5, lanes 6–8). This phosphoprotein persisted in NFB4 cells maintained in DM for 72 h (data not shown). Thus, IGF-dependent myogenic differentiation in C2C12 and NFB4 cells was correlated with a common array of phosphotyrosine-containing proteins. By contrast, NFB4 cells in GM and DM exhibited specific differences in tyrosine phosphorylation relative to wild-type C2C12 cells.

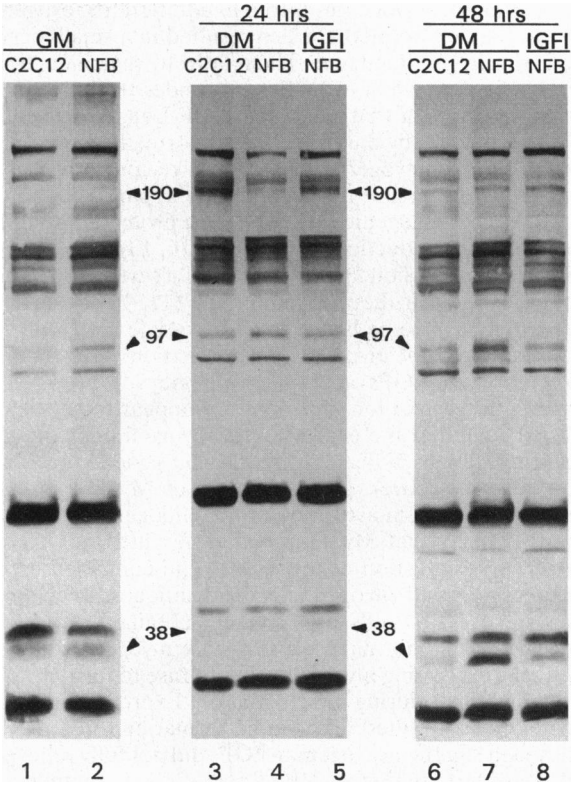

FIG. 5. Western analysis of phosphotyrosine-containing proteins in NFB4 and C2C12 cells. Protein extracts (60 μg) from NFB4 and C2C12 cells cultured under different conditions were separated by SDS/PAGE, transferred to Immobilon-P membranes, and incubated with antibody 6G9 to phosphotyrosine. Lane 1, C2C12 in GM; lane 2, NFB4 in GM; lane 3, C2C12 in DM for 24 h; lane 4, NFB4 in DM for 24 h; lane 5, NFB4 in serum-free medium supplemented with IGF-I, for 24 h; lane 6, C2C12 in DM for 48 h; lane 7, NFB4 in DM for 48 h; and lane 8, NFB4 in serum-free medium supplemented with IGF-I, for 48 h. The positions of proteins of 190, 97, and 38 kDa are indicated.

DISCUSSION

Myoblast differentiation induced by removal of serum is correlated with increased accumulation of IGF-II and myogenin mRNAs and downregulation of gene products associated with proliferation, such as Jun, Fos, and Myc. By contrast, nondifferentiating NFB4 muscle cells failed to express either IGF-II or myogenin transcripts at detectable levels and continued to accumulate the mRNAs encoding the protooncogene products upon serum withdrawal. Supplying either IGF-I or myogenin to NFB4 cells activated the expression of the nonsupplied one and resulted in a partially differentiated phenotype. However, the differentiated phenotype of NFB4 cells following treatment with exogenous IGFs was aberrant. Approximately 50% of the cells responded by biochemically differentiating, as determined by myosin heavy chain expression, but multinucleated myotubes were not formed. The partial rescue of the differentiated phenotype may be due to the continued expression of Jun, Fos, and Myc. These gene products have been shown to be antagonistic to myogenic differentiation by inhibiting the activity of the helix–loop–helix transcription factors (33–35). Clearly, myogenin and MyoD retained some function in the presence of the protooncogene products but were not able to promote fully normal differentiation. Thus, the balance between proliferation- and differentiation-promoting factors has been shifted toward proliferation in NFB4 cells, and simply supplying molecules known to be involved in controlling differentiation does not completely rescue the mutant phenotype.

Activation of myogenin through addition of exogenous IGF-I to the culture medium also resulted in a small increase in IGF-II mRNA abundance. The relatively weak induction of IGF-II gene expression in NFB4 cells under these conditions may reflect the fact that exogenous IGF-I appears to inhibit IGF-II production by muscle cells that normally occurs in low serum (3). That myogenin and IGF-II reciprocally activate each other's expression may explain the apparently paradoxical results that myogenic differentiation promoted by IGF-II treatment led to induction of myogenin (6, 13), but increased IGF-II gene expression upon withdrawal of serum appeared to follow activation of the myogenin gene (11, 16). IGF-II and myogenin appear to be linked in an autocrine loop, and both may be required for myogenic differentiation.

Whereas both IGFs and myogenin may be required for myogenic differentiation, this does not appear to be the case for MyoD. Neither the expression of myogenin nor the presence of exogenous IGFs activated MyoD gene expression in NFB4 cells. Moreover, as transfection of MyoD expression constructs resulted in activation of the endogenous myogenin gene, it is likely that MyoD affects differentiation of NFB4 cells through activation of myogenin. The combined expression of MyoD and myogenin had no unique effect on the phenotype of NFB4 cells, unlike results obtained with BC_3H1 cells. These cells normally express only myogenin and, like NFB4 cells expressing myogenin, do not fuse to form multinucleated myotubes during differentiation. Expression of MyoD in BC_3H1 cells resulted in myotube formation (36).

Although supplying exogenous IGFs did not fully relieve the block to differentiation in NFB4 cells, IGF-I treatment did result in secretion of IGFBP-5 to a level comparable to that in wild-type cells. The role of IGFBP-5 in myogenic differentiation is not clear, as it has recently been shown to augment the stimulatory effect of IGF-I on myogenic differentiation but inhibit that of IGF-II (J. Florini, personal communication). IGF-I treatment also resulted in the appearance of a wild-type array of phosphotyrosine-containing proteins. The phosphorylation of a 190-kDa protein that occurred in C2C12 cells within 24 h of exposure to DM did not occur in NFB4 cells unless the cells were treated with IGFs. The role of this protein in the signal transduction cascade has yet to be determined but may represent a substrate for the IGF-I receptor, as was recently described for the insulin receptor (37). Moreover, additional proteins at 97 and 38 kDa reacted more strongly with the anti-phosphotyrosine antibody in NFB4 than C2C12 cells, and the phosphorylation state of both proteins returned to wild-type levels in response to IGF-I. That these phosphoproteins differed in their pattern of expression suggests that they participate at different points in the phosphorylation cascades. However, their size makes both of them candidate members of the mitogen-activated protein (MAP) kinase family, specifically p97, ERK-3, or ERK-5, and p38, osmotic stress activated kinase, respectively (38–40). That these MAP kinases may be involved in the balance between proliferation and differentiation in muscle cells is suggested by the work of Campbell *et al.* (41) showing that the well-characterized MAP kinases ERK-1 and ERK-2 do not appear to be involved in controlling proliferation or differentiation in MM14 muscle cells.

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